"PHYTOCHEMICAL & BIOLOGICAL SCREENING OF *THUJA OCCIDENTALIS* L. EXTRACTS"

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In Partial Fulfillment of the Requirements for the Degree of

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CANDIDATE'S DECLARATION

I/We Shagun Kapoor(2K22/MSCCHE/37) and Sarbani Ganguly(2K22/MSCCHE/35) hereby certify that the work which is being presented in the dissertation entitled "**Phytochemical and Biological Screening of *Thuja occidentalis* L. leaf extracts"** in partial fulfilment of the requirements for the award of the Degree of Master of Science in Chemistry, submitted in the Department of Applied Chemistry, Delhi Technological University is an authentic record of our work carried out during the period from 2022 to 2024 under the supervision of Prof. Archna Rani and co-supervision of Prof. Vidhu Aeri.

The matter presented in the dissertation has not been submitted by us for the award of any other degree of this or any other Institute.

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Certified that **Shagun Kapoor (**2K22/MSCCHE/37)& **Sarbani Ganguly** (2K22/MSCCHE/35) have carried out their research work presented in this dissertation entitled **"Phytochemical and Biological Screening of *Thuja occidentalis* L. leaf extracts"** for the award of **Master of Science in Chemistry** from Department of Applied Chemistry, Delhi Technological University, Delhi, under our supervision. The dissertation embodies results of original work, and studies are carried out by the students themselves and the contents of the dissertation do not form the basis for the award of any other degree to the candidates or anybody else from this or any other University/Institution.

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Abstract:

The present research includes a comprehensive examination of the Phytochemical and Biological Screening of *Thuja occidentalis* L. leaf extract, a botanical medicine derived from the white cedar or northern white cedar tree. *T. occidentalis* has a long and significant history in traditional medicine, as it is believed to possess medicinal characteristics. The main aim of this study is to determine the chemical makeup of the extract from the plant *T. occidentalis*. The chemical composition was determined through the application of different analytical methods, including chromatography, mass spectrometry and computational methods such as molecular docking.

These techniques revealed a wide range of plant-derived components, such as terpenoids, flavonoids, phenolic compounds, and essential oils. The phytochemical analysis was performed as well and pharmacological properties were also evaluated using tests that targeted some biological systems, such as *Bacillus Cereus* and molecular docking analysis was also performed. The results suggest that the extract has notable pharmacological potential, as it exhibits antibacterial effects against a pathogen. These findings enhance the comprehension of *T. occidentalis* as a therapeutic substance and could aid in its incorporation into pharmaceutical uses. In summary, this study highlights the significance of conducting more research on the therapeutic advantages of *T. occidentalis* extract and its potential use in contemporary medicine.

Keywords: *Thuja Occidentalis* L., Chromatography, Phytochemicals, Molecular docking.

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List of Abbreviations

Thuja occidentalis- T. occidentalis

GPP - Geranyl pyrophosphate

EOs - Essential oils

MIC - Minimum inhibitory concentration

BC - *Bacillus cereus*

OD - Optical density

ZOI - Zone of Inhibition

GC - Gas chromatography

GCMS - Gas chromatography-mass spectrometry

Chapter – I INTRODUCTION

Introduction

Thuja occidentalis Linn., also referred to as white cedar or northern white cedar, is a type of evergreen tree that is native to North America. It is most commonly found in areas with high levels of moisture, such as swamps and wetlands(Dosoky and Setzer, 2021). Indigenous civilisations have historically used different sections of *Thuja occidentalis* for medicinal purposes, ascribing it to a range of therapeutic characteristics. Currently, there is a renewed interest in botanical medicines, and the extract from the *T. occidentalis* plant is attracting attention due to its possible pharmacological effect(Bhargava et al., 2022).

This dissertation seeks to close this divide by carrying out a thorough analysis of the Phytochemical and Biological Screening of *T. occidentalis* leaf extracts. The main objective is to analyse the chemical components found in the extracts and assess their pharmacological effects through meticulous experimentation. The characterization of *T. occidentalis* extracts will include the application of numerous analytical procedures including chromatography, and mass spectrometry. These techniques will facilitate the detection and measurement of phytochemicals found in the extract, providing insights into its chemical intricacy(Sonia et al., 2020). Simultaneously, pharmacological studies will be performed to evaluate the extract's potential therapeutic effects on some biological systems. The mind map of this dissertation is depicted in Figure 1.

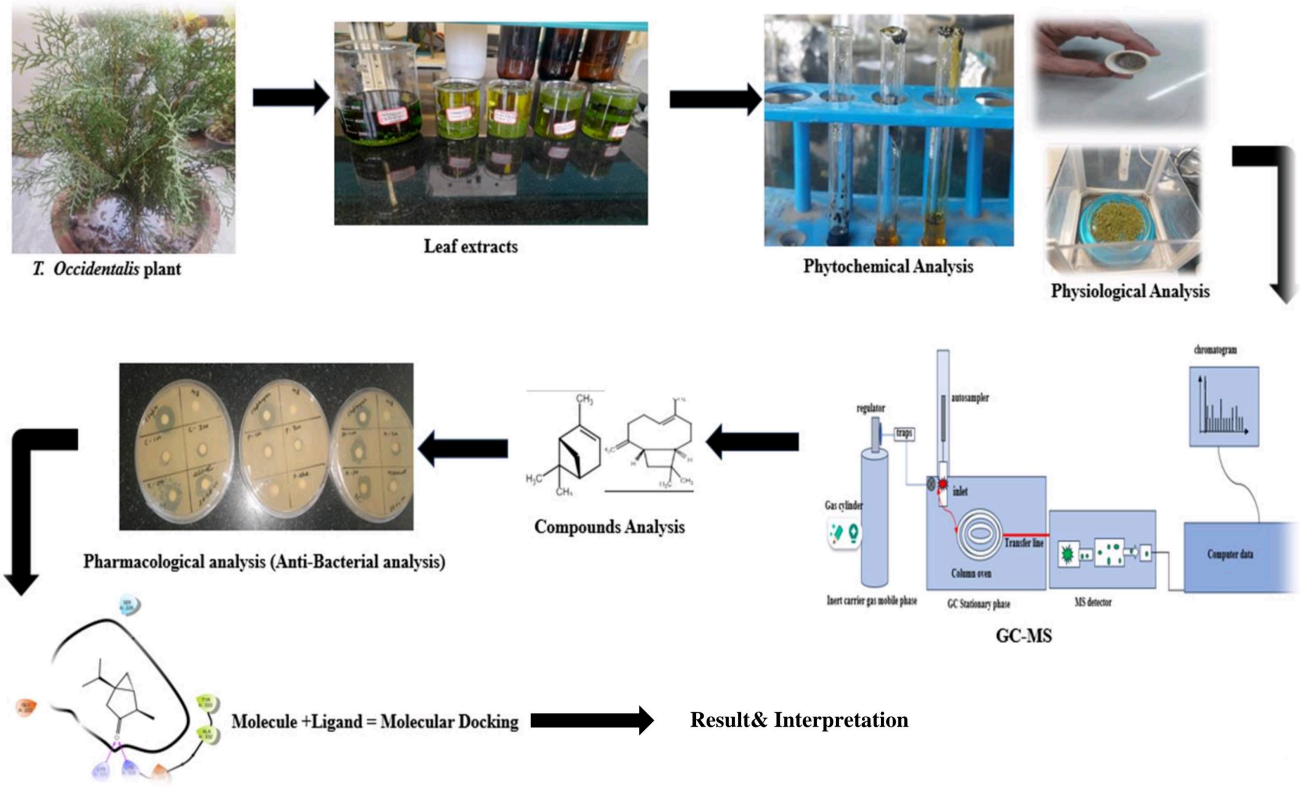


Figure 1 Mind map of this dissertation

1.1 Historical Background

Thuja occidentalis L. possesses a significant historical heritage closely interconnected with the indigenous cultures of North America(Dosoky and Setzer, 2021). The indigenous populations of the area, including the Algonquin, Iroquois, and Ojibwa, have deeply respected *T. occidentalis* for its medicinal attributes and integrated it into their customary therapeutic methods. (Bhargava et al., 2022).

The foliage, branches, outer covering, and even the sticky substance produced by the tree were employed in medicinal treatments targeting a wide array of health issues(Duhan et al., 2013). The extensive use of *T. occidentalis* in indigenous healing practices emphasises its importance as a botanical treatment and showcases its cultural significance(Dubey et al., 2017).

Despite its extensive historical use and acknowledgement in traditional and early modern medicine, lost its popularity over time due to the rise of synthetic medications in the 20th century(Dubey et al., 2017). In recent years, there has been a renewed interest in botanical treatments, leading to a rebirth of scientific investigation into the therapeutic properties of *T. occidentalis* extract and renewed research of its historical origins.

1.2 Research Objective

This research aims to thoroughly analyse the chemical makeup of *T. occidentalis* leaf extracts in different solvents, carry out Physiological & Phytochemical analysis and clarify the therapeutic value of *T. occidentalis* extract by using a combination of analytical techniques.

1.3 Research Gap

Although *T. occidentalis* extract has been used traditionally for a long time and there have been reports of its medicinal effectiveness, there is a dearth of extensive scientific research on its chemical composition and pharmacological actions(Tsiri et al., 2009). The lack of knowledge in this area prevents its full integration into contemporary pharmaceutical applications and restricts our comprehension of its therapeutic characteristics(Nelson, 2019). The intricate nature of *T. occidentalis* extract, which consists of several phytochemical elements that may interact synergistically or antagonistically, poses difficulties in determining the primary bioactive molecules responsible for its observed pharmacological effects(Tlili and Sarikurkcud, 2020).

1.4 Research Significance

This observation has important consequences for scientific comprehension and practical applications in the realms of pharmacology and natural medicine(Dosoky and Setzer, 2021). This work enhances the existing information about herbal treatments by thoroughly analysing the chemical makeup of *T. occidentalis* extract(Bhargava et al., 2022). Furthermore, the assessment of pharmacological activities linked to the extract of *T. occidentalis* fills an important void in existing scientific literature(Sonia et al., 2020).

The purpose is to gather factual information that either supports or disproves the traditional and anecdotal assertions about its medical characteristics. Empirical validation is crucial in order to prove the credibility and dependability of *T. occidentalis* extract as a possible

medicinal agent(Bhargava et al., 2022). Moreover, the results of this study have practical ramifications for the advancement of pharmaceuticals and healthcare(Jasuja et al., 2013).

1.5 Research limitations

The chemical makeup of *T. occidentalis* extract is complicated, hence it's possible that some aspects of it were not fully captured in this study(Sonia et al., 2020).*T. occidentalis* extract's chemical makeup and pharmacological properties can change based on a number of variables, including the plant source, the extraction technique, and the solvent employed(Jasuja et al., 2013). Although this study sheds light on the possible pharmacological properties of *T. occidentalis* extracts, more clinical research is required to confirm its effectiveness, safety, and therapeutic value in human populations.

Chapter – II REVIEW OF LITERATURE

2.1 Theoretical Framework

The conceptual framework that directs the design of the study, the methodology, and the interpretation of the data is provided by the theoretical framework that underpins the research on the characterization and pharmacological activity of *T. occidentalis* extract (Duhan et al., 2013). Within this section, in order to investigate the chemical composition of *T. occidentalis* extract as well as its bioactive qualities, phytochemistry and pharmacognosy offer theoretical frameworks that can be utilized (Sonia et al., 2020).

2.1.1 Botanical Description and Therapeutic Use

The Eastern North American white cedar, scientifically known as *T. occidentalis*, is a natural tree species. One other name for it is American Arbor Vitae. They are cultivated in Brazil and Europe for their aesthetic appeal. The tree's maximum height is between fifteen and twenty meters. Trees of this species are monoecious. *T. occidentalis* is distinguished by its pyramidal shape, which is characterized by dense, scale-like leaves that are grouped in sprays that are flattened(Maia and Moore, 2011). There is a unique aromatic perfume that emanates from the foliage when it is crushed, which contributes to its recognition and the traditional applications that it has. It is most commonly seen growing in damp habitats such as swamps, wetlands, and rocky slopes, and it thrives in conditions that are classified as cool temperate (Nazir et al., 2016). Leaves of T. occidentalis are shown in Figure 2.1

Figure 2.1 *T. occidentalis* leaves

Traditional therapeutic use

In traditional Chinese medicine, the leaves and stems of *Thuja orientalism* were used to cure a variety of conditions, including fevers, bleeding, mental problems, insomnia, and irregular heartbeats. Also, according to TCM, you should steep seven days of fresh cedar leaves in a 60% alcohol solution if you want your hair to grow faster. The mixture was massaged into the bald spots three times daily.

Western herbalists employ cedar leaf oil for a variety of purposes, including as an emmenagogue, an abortion inducer, a digestive aid, a vermifuge, and a diuretic. Its topical application cured thrush, ringworm, and warts in the anal and vaginal regions, as well as arthritic and rheumatic pains. Native Americans employed cedar leaf preparations for a variety of medical purposes, including headache relief and scurvy prevention. The cedar tree, scientifically known as Arbor Vitae, was once used to treat or prevent scurvy due to the high vitamin C content of its leaves and twigs(Aswath, 2014).

Modern therapeutic use Moreover, multiple studies conducted recently discovered that the herb had anti-inflammatory characteristics. Thuja essential leaf oil has a long history of use as

a treatment for a wide variety of illnesses, including cancer, intestinal worms, and fungal infections(Thakur, 2016). Furthermore, there is substantial evidence that secondary metabolites found in plants can effectively combat germs. Many of the antiviral compounds included are either out-of-date or German-published results, but they have a great in vitro healing affinity for the herpes simplex virus.

Research has shown that *T. occidentalis* can inhibit the growth of several viruses, including influenza A, HIV-1, and *Verruca vulgaris*. Furthermore, *T. occidental possesses* the capacity to supply antioxidants. This ethanolic fraction of Thuja contains antidiabetic characteristics and is an excellent treatment for diabetic nephropathy. An earlier experiment also found that the herb has anti-atherosclerotic properties. Furthermore, there is promising evidence that the essential oil of this plant can treat polycystic ovarian syndrome. It helps with gynaecological issues and works as an emmenagogue to prevent periods from getting obstructed by reducing period symptoms like bloating, cramping, nausea, and exhaustion.

T. occidentalis possesses immunomodulatory effects when mixed with other herbs. A homoeopathic medicine that uses the leaves of the *T. occidentalis* tree is named Thuja. The most widely used homoeopathic reference book recommends Thuja for the following conditions: headaches, vertigo, emotional depression, restlessness, itching or pain on the scalp, (Thakur, 2016) difficulty swallowing, excessive thirst, frequent urination (with frothy or cloudy urine), disturbed sleep caused by stress or anxiety, and nighttime fever and chills.

As a foundation note in aromatherapy, cedar leaf oil imparts a scent that lingers after adding it to incense or perfume mixtures. This medicine is recommended for individuals experiencing anxiety, asthma, bronchitis, or a common cold because of its calming or sedative properties. And for those pesky zits and dandruff, aromatherapists recommend cedar leaf oil.

2.1.2 Composition of *T. occidentalis*

Due to the possible pharmacological relevance of the leaf extract of *T. occidentalis*, the chemical makeup of the extract is the topic of intensive research (Bhargava et al., 2022). An examination of the methanolic extract reveals that it contains a diverse assortment of bioactive chemicals, such as terpenoids, flavonoids, phenolic compounds, and essential oils, all of which contribute to the extract's medicinal effects (Biswas et al., 2011).

Terpenoids, which are often referred to as terpenes, are one of the most important families of chemicals that may be discovered in an extract of *T. occidentalis*(Dubey et al., 2017). These chemical compounds are distinguished by the wide variety of structures and biological activities that they exhibit (Duhan et al., 2013). Furthermore, terpenoids are thought to play a key part in the traditional therapeutic use of the plant, in addition to contributing to the distinctive perfume of the plant (Biswas et al., 2011).

In addition to terpenoids, the extract of *T. occidentalis* contains flavonoids, which are an important category of compounds (Duhan et al., 2013). These polyphenolic chemicals are well-known for their antioxidant qualities, which allow them to scavenge free radicals and shield cells from the damaging effects of oxidative stress (Dubey et al., 2017). Furthermore, flavonoids have been shown to possess anti-inflammatory and antibacterial properties, which makes them significant contributions to the therapeutic potential of the extract(Jenifer, 2023).

T. occidentalis, the methanolic extract contains a significant number of phenolic components, such as phenolic acids and tannins, among other phenolic compounds (Jasuja et al., 2013). The potential of the extract to counteract oxidative stress, inflammation, and microbial infections

is a direct result of the presence of these chemicals, which contain antioxidant, anti-inflammatory, and antibacterial characteristics (Biswas et al., 2011).

The essential oils that are produced by *T. occidentalis* contain volatile aromatic chemicals, which are responsible for the plant's distinctive aroma and the medical properties that it possesses (Jasuja et al., 2013). These oils have qualities that make them antibacterial, antifungal, and analgesic, which allows them to enhance the therapeutic potential of the extract in the treatment of a variety of diseases(Dubey and Barta, 2009). The composition of T. occidentalis is described in Figure 2.2.

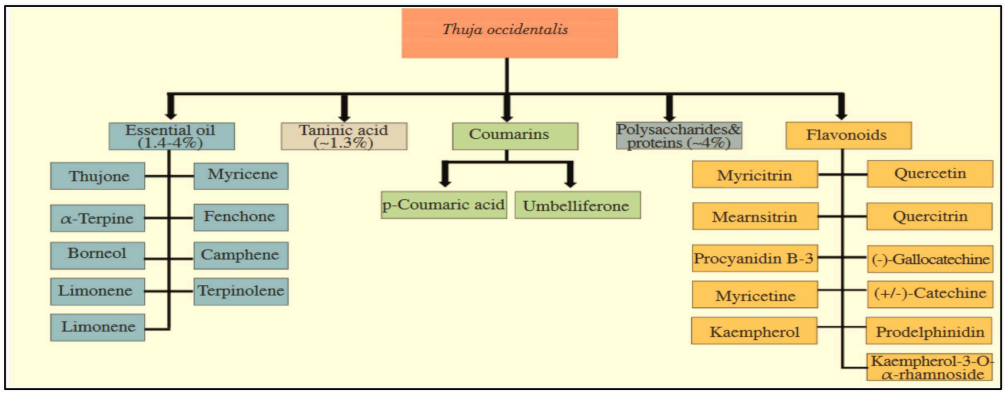


Figure 2.2 *T. occidentalis* composition

2.1.3 Pharmacological Activities

The methanolic extract of *T. occidentalis* demonstrates a wide variety of pharmacological activity, which makes it a promising option for therapeutic treatments in a variety of health disorders (Bhargava et al., 2022) as depicted in Figure2.3. The extract possesses powerful antibacterial characteristics and has the potential to offer therapeutic benefits in the treatment of bacterial infections (Bhargava et al., 2022). As a result of the fact that its antimicrobial effects extend to antifungal qualities, it is also a potential candidate for treating fungal infections. The methanolic fraction extract of *T. occidentalis* has the ability to modify the immune system, thereby increasing immunological responses against infections while simultaneously regulating immune function in order to prevent conditions related to the immune system or excessive inflammation (Jasuja et al., 2013).

The extract exhibits analgesic qualities, meaning that it alleviates pain through mechanisms that may involve the modulation of pain signalling pathways or the suppression of inflammatory mediators (Jasuja et al., 2013). The extract of *T. occidentalis* had been shown to have vasodilatory effects on cardiovascular parameters, which results in the possible improvement of blood flow and cardiovascular function (Bhargava et al., 2022).

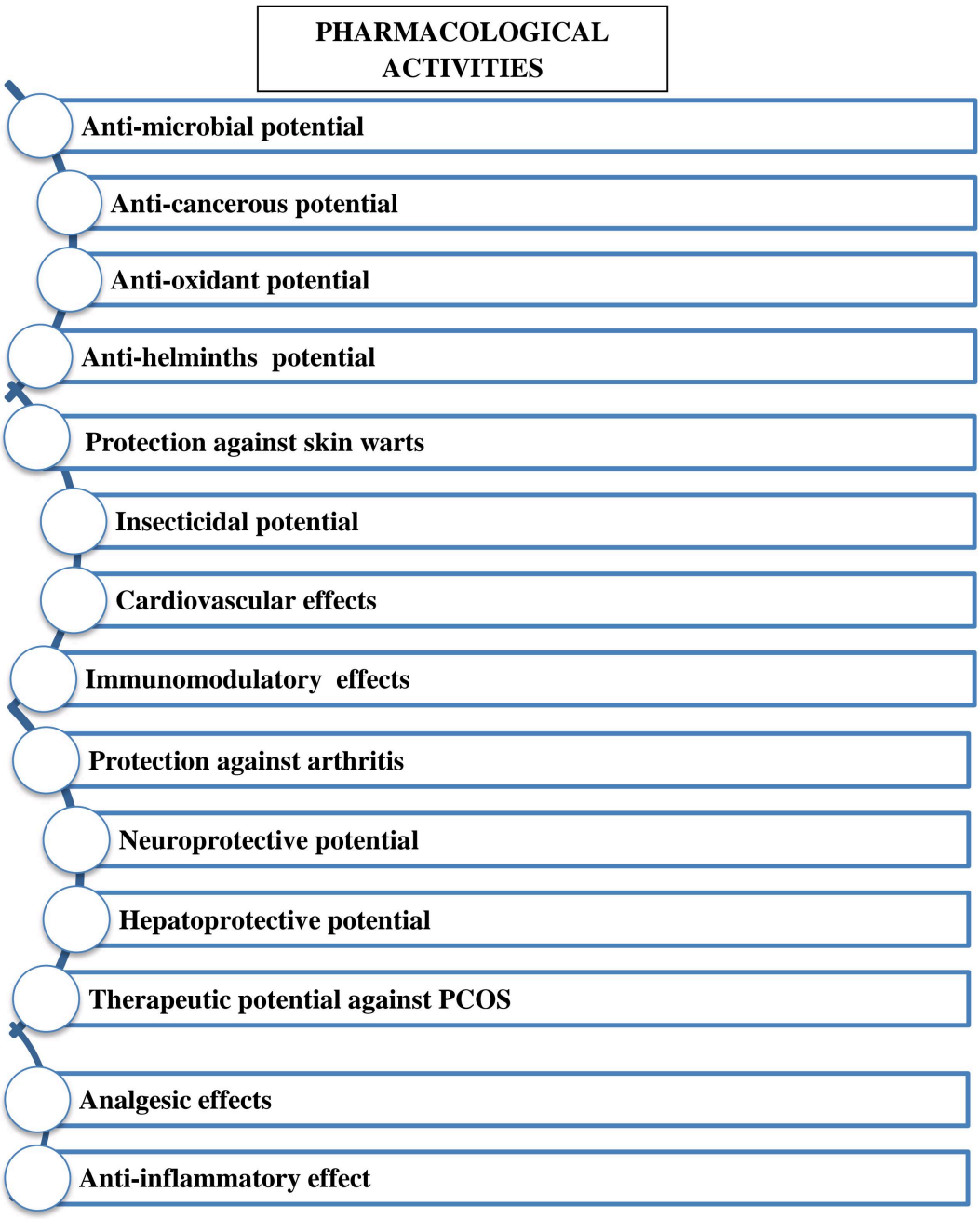


Figure 2.3 Biological activity shown by *T. occidentalis*

2.1.4 Biosynthesis of Thujone and its properties

Terpenoids, steroids, flavonoids, and polysaccharides are all components that have been extracted from *T. occidentalis*, as indicated by previous phytochemical study (Guleria et al., 2008). It is believed that thujone is the main bioactive component in the essential oil of *T. occidentalis* species (Bhargava et al., 2022). The epimeric forms of this monoterpene include both (+)-β-thujone and (−)-α-thujone. Thujone is referred to by its IUPAC designation, which is 4-Methyl-1-(propane-2-yl) bicyclo[3.1.0] hexan-3-one (1S, 4R, 5R for short).

To make thujone and all monoterpenes, the enzyme geranyl diphosphate synthase converts dimethylallyl pyrophosphate and isopentenyl diphosphate into geranyl diphosphate, also known as geranyl pyrophosphate (GPP). The enzyme sabinene-synthase creates the initial monoterpene, sabinene, from GPP (Bhargava et al., 2022). Making cissabinol (Salvia officinalis) or trans (T. plicata) from sabinene is the next metabolic step. Thujone synthesis relies on NADPH to carry out a stereoselective reduction of sabinone (Dubey et al., 2017). The steps to produce thujone are depicted in Figure 2.4.

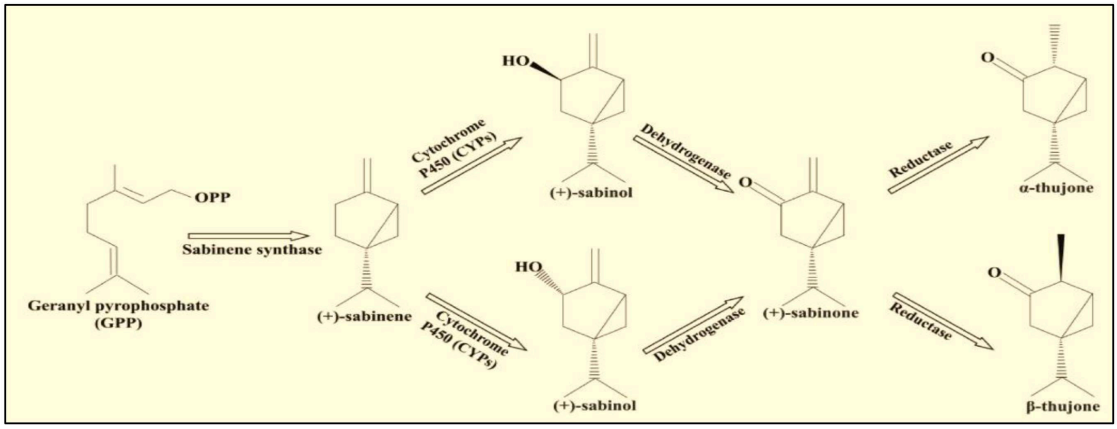


Figure 2.4 The biosynthesis of thujone from geranyl diphosphate (Thakur, Sobti, & Kaur, 2023)

Thujone is used for a wide range of tumours, including adenocarcinomas, glioblastomas, ovarian malignancies, and cutaneous tumorigenicity (Jasuja et al., 2013). Thujone induces the abnormal expression of ATF6 phosphorylation in the endoplasmic reticulum as a reaction to endothelial stress (Dubey et al., 2017).

2.2 Presence of Thujone in other plants

The essential oil component thujone is produced by a variety of plants. Plants that contain thujone have numerous medicinal and culinary applications, including the treatment of intestinal viruses, making wine, tonics, carminatives, and astringents. Additionally, these plants are effective against worms. Some plant families have more thujone than others; these include the Asteraceae and the Cupressaceae. Several plants contain floral thujone, which has significant culinary uses.

The main components of essential oils derived from plants that contain thujone include wormwood *(Artemisia absinthium),* mugwort *(Artemisia vulgaris),* sage *(Salvia officinalis),* clary *(Salvia sclarea),* tansy *(Tanacetum vulgare), and* white cedar *(T. occiden*talis*).* The plant names and the part of plants in which thujone is observed are mentioned in Table 2.1. It

is often believed that plants containing thujone have the following pharmacological effects, albeit the specific components of oils produced from these species can differ. **1)** a remedy for opium and other CNS depressants; **2)** teratogenic; **3)** Anthelmintic; **4)** hallucinogenic;**5)** Additional properties;

Table 2. 1 Description of thujone in other plants

Plant Name	Parts of Plant
Wormwood	Stems, roots, leaves
Sage	Leaves
Mugwort	Roots &stems
Clary	Leaves, flowers, stems & seeds
Thuja	Leaves
Tansy	Stems
Cypress	Leaves, cones, wood, branches
Oregano	Leaves, stems, flower buds
Juniper	Berry, branches, leaves
Menthe	Leaves, flowers, stems, bark, seeds
Lavender	Buds, leaves &stems

2.3 Chemical Structure of Thujone

Thujone is an inherent chemical present in particular plants, specifically classified as a monoterpene ketone. There are two variants, classified as α-thujone and β-thujone. Essential oils (EOs) extracted from plant families such as Cupressaceae, Lamiaceae, and Asteraceae prominently contain Thujone as a constituent. Factors such as plant variety, genetics, ambient circumstances, and the specific plant part that yields the essential oil influence the levels and abundance of thujone in these plants.

Sage and white cedar often contain more α-thujone than tansy and wormwood, which are more β-thujone-rich. Thujones in A. absinthium oils ranged from 36.8% to 39.6% for α-thujone and from 10.1% to 69.9% for β-thujone, according to research by Nguyen and Ne'meth (2016).In most cases, one isoform is more common than the others, although this does not appear to be the case across all species.

Thujone is colourless and has a structure similar to a terpene. Its molecular formula is $C_{10}H_{10}O$. The compound exhibits a chemical structure that closely resembles camphor and is comprised of two distinct forms: laevorotatory (-) α-thujone and dextrorotatory (+) ß-thujone. α-thujone

is more abundant than ß-thujone. As the structures and isomers so of thujone are depicted in Figure 2.5.

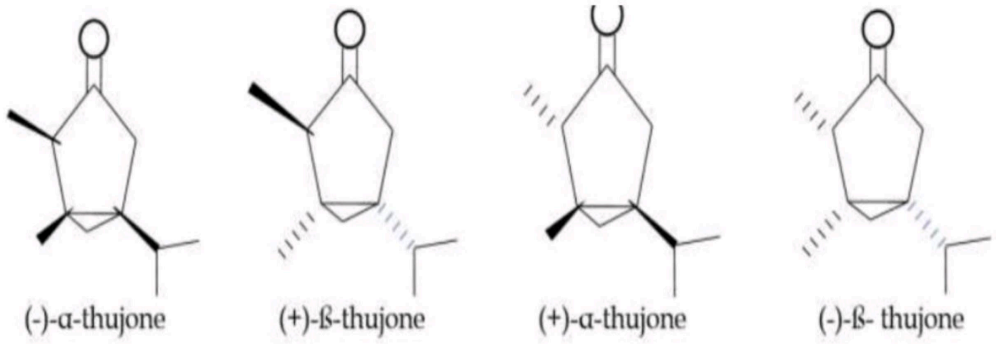


Figure 2.5 The structures and isomers of α- and β-thujone (Swapnil Pal, 2023)

Isomers of Thujone:

Thujone can produce four stereoisomers due to its bicyclo [3.1.0]-hexanone core structure, which also includes methyl and cyclopropyl stereocenters. According to earlier studies, stereoisomers are diastereomers that are epimers at the methyl position and share the same stereochemistry at the cyclopropane position (Williams, 2016). Many plant species, such as *Salvia officinalis L.* and *Thuja plicata*, have had their biosynthetic pathways for the common (+)-β and (-)-α stereoisomers proposed. Most of the stereoisomers in these plants are thought to originate from (+)-sabinone. predictable and possible isomers of thujone are shown in Figure 2.6 and their stereochemistry can be observed through Figure 2.7.

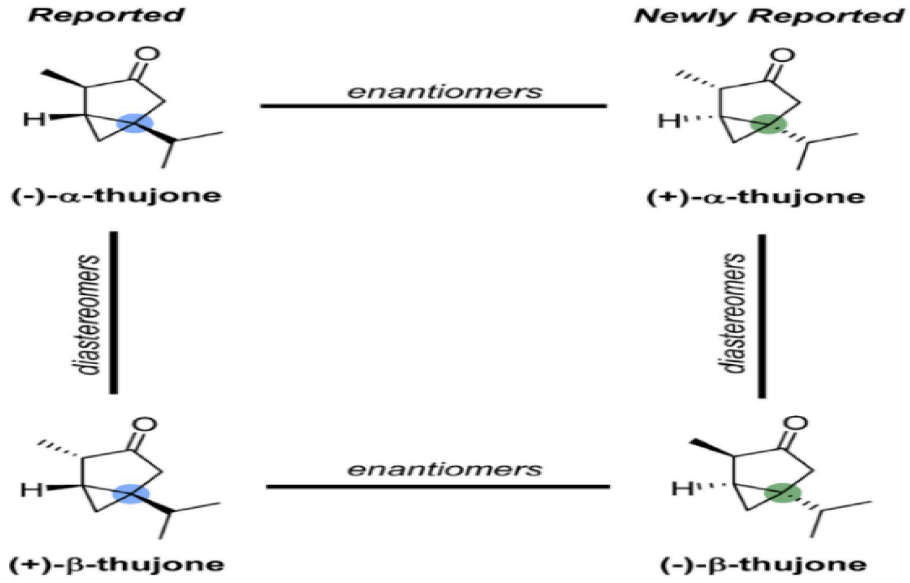


Figure 2.6 Possible stereoisomers of thujone (Williams, 2016)

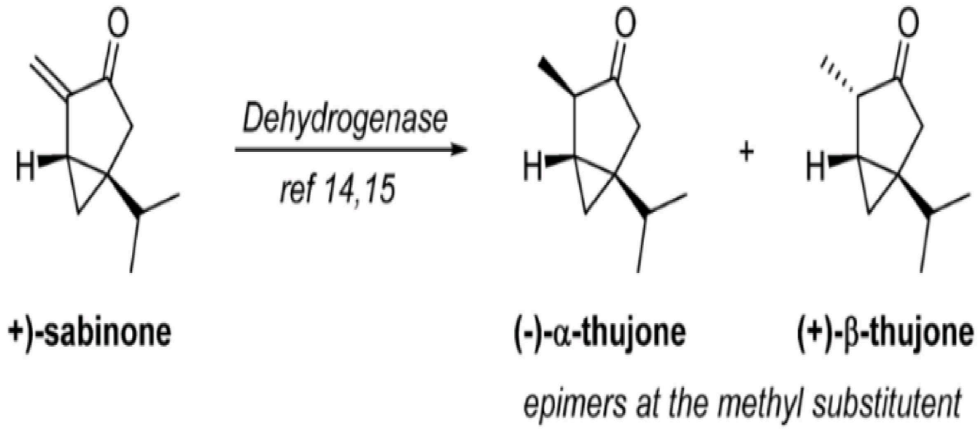


Figure 2.7 The stereochemistry in the noted thujone stereoisomers: a possible biological origin (Williams, 2016)

The majority of the present studies on thujone in plants focus on the presence of (−)-α-thujone and (+)-β-thujone, but they have not looked into the possibility of the equivalent stereoisomers, (+)-α-thujone and (−)-β-thujone (Jack D. Williams, 2016). Factors including harvest timing, country of origin, and genotype can have a major impact on the enantiomeric compositions of phytochemicals. The exact enantiomeric compositions of thujone in different plants have not been well studied, despite the fact that many plants produce distinct enantiomers of natural compounds.

2.4 Methods of Extraction of Essential Oil

Several techniques, such as enfleurage, steam extraction, solvent extraction, hydrodistillation, and supercritical fluid extraction, were utilized in order to extract essential oils from the plant material (Dosoky and Setzer, 2021). For this investigation, the following methodologies were explored:

1. **Steam Distillation**: A technique used to separate volatile compounds from non-volatile compounds in a mixture by using steam to vaporize the volatile compounds, which are then condensed and collected separately.

2. **Hydrodistillation**: This method involves using water and heat to vaporize the volatile compounds present in the plant material, which are then condensed and collected as essential oil.

3. **Microwave-Assisted Hydrodistillation**: A recent technique that quickly extracts essential oils from plant material by heating it in a microwave oven, resulting in shorter extraction or distillation times.

   The **Clevenger Apparatus** is a specialized piece of laboratory glassware used for the hydro distillation of essential oils from plant materials. It consists of a flask connected to a condenser, which leads to a graduated tube. During operation, steam or boiling water extracts volatile compounds, which then condense and collect in the graduated tube. This setup allows the separation of essential oils based on their immiscibility with water. The Clevenger Apparatus is extensively used in pharmaceutical, cosmetic, and food industries for extracting and analysing essential oils, providing a simple yet

effective method to obtain high-purity oil samples (Manish et al., 2023).The apparatus description is given in Figure 2.8.

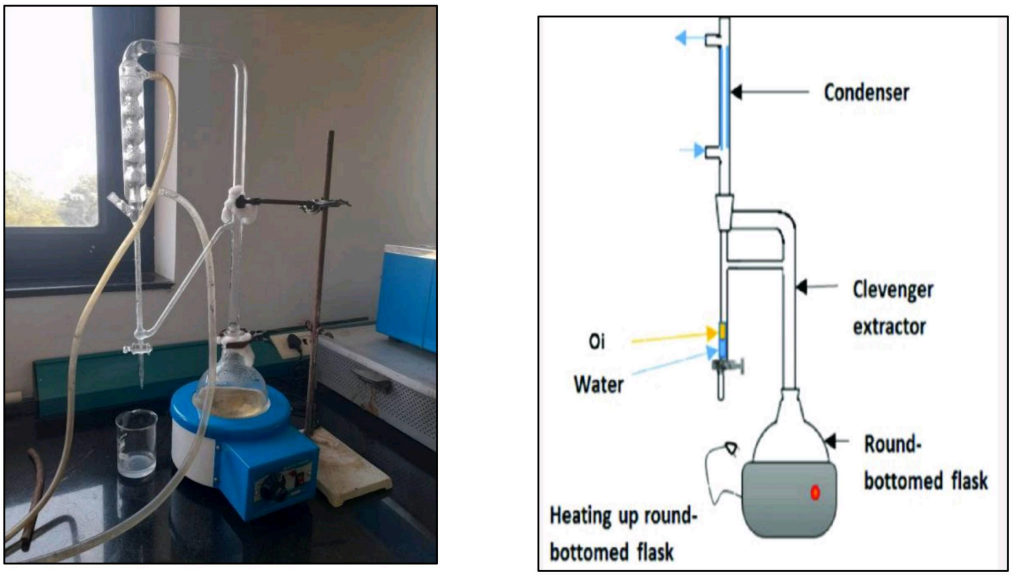


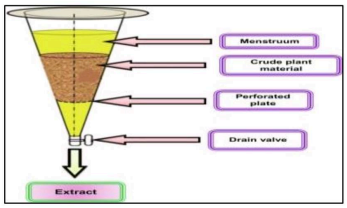
Figure 2.8 Clevenger Apparatus

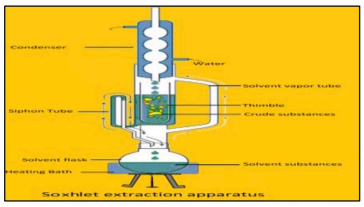
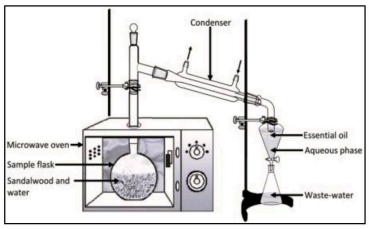
2.5 Crude extraction methods

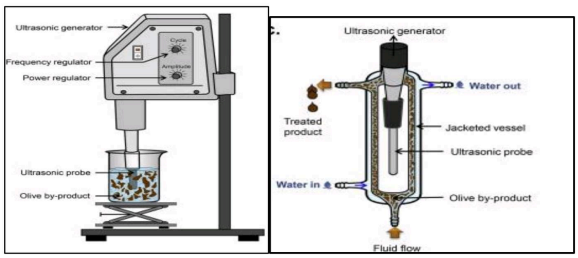
Different methods for the extraction of crude extract are tabulated in Table 2.2

Table 2.2 Methods of extraction of Crude extract

S. No.	METHODS
1)	Maceration To make a complete soak, add some finely crushed leaves, stem bark, or root bark to a jar with some solvent. Recap and refrigerate for at least three days. To ensure complete extraction, stir or shake the mixture periodically. Filtration or decantation is used to separate the marc from the micelle after extraction. The process of separating micelles from fluid involves evaporating them in an oven or water bath.  **Advantage:** it requires a simple container for extraction, cheap, easy and ideal for thermolabile plants, generally used for less soluble solvents.

	Disadvantage: The extraction process takes a lengthy time, and low extraction efficiency amounts of organic solvent are used.
2)	Decoction This water-based treatment extracts medicinal plant active ingredients. Liquid preparation is made by boiling plant material with water. Most plant material is pulverized or broken down. Ayurvedic extracts are often made this way. Beginning with 1:4 or 1:16, the crude drug-to-water ratio is fixed. Boiling reduces the volume to 25% during extraction.  **Advantage:** A heat-insensitive approach for extracting water-soluble components, no expensive equipment needed. **Disadvantage:** Large amounts of organic solvent are used and low extraction efficiency.
3)	Percolation This is the preferred approach for extracting phytochemicals from fluid extracts. Our percolator is thin and cylindrical in shape, with wide openings at both ends. Put the dried, powdered plant material and the extraction solvent in a clean container. Add more solvent and let it sit for four hours. After sealing the percolator's bottom, let everything sit for 24 hours. Careful addition of the solvent from the top guarantee's full dissolution of the medication. To speed up the slow flow of liquid, open the lower percolator. Solvent was continuously pumped into the therapeutic material as it was driven through by gravity. In order to stop adding solvent, the level had to reach 75% of the starting volume. Using filtration and decanting, the extract is isolated. After expressing the marc, add the final solvent to get the volume you need.  **Advantage:** faster than maceration process, thermolabile components can be extracted. **Disadvantage:** takes longer time than soxhlation, extra solvent needed
4)	Soxhlet extraction The optimal hot solvent technique for continuous solid extraction. A Soxhlet glass extractor is utilized. Complete with a condenser on top, a spherical bottom flask, an extraction chamber, and a siphon tube. A thimble, consisting of strong filter paper or clean

	fabric, is a porous pouch that holds the dried, crushed, and finely powdered plant material. Once the bottom flask is filled with extraction solvent, place the thimble in the extraction chamber. The heat from the bottom flask evaporates the solvent, which is then delivered to the extraction chamber. Concentrating and releasing medication is now as easy as pressing a finger. As soon as the solvent and plant material reach the top of the siphon in the extraction chamber, they are returned to the flask. After the solvent has emptied the extraction chamber, the process is repeated until no more residue is left. Even partially soluble or insoluble plant materials can be effectively processed using this method.  **Advantage:** large amounts of medication can be extracted with less solvent, and Heat-resistant plant materials can be used it. **Disadvantage:** it blocks agitation, unsuitable for thermolabile materials.
5)	Microwave-assisted extraction This is an advanced medicinal plant extraction method. The method combines dipole rotation and ionic transfer to displace charged ions in solvents and drugs. It uses 300 MHz–300 GHz electromagnetic radiation. The flowchart shows the steps: Microwave radiation heats moisture, causing evaporation, cell wall swelling, cell rupture, and phytoconstituent leaching. The dielectric constant affects solvent choice, dissipation factor, and microwave power.  **Advantage:** uses less solvent and extraction time, Equipment is cheap and solvent recovery is high using this process. **Disadvantage:** Processing is prohibited with non-polar solvents; Volatile solvents reduce process efficiency.

6)	Ultrasound-assisted extraction Using ultrasonic waves within the 20KHz to 2000KHz frequency range, this treatment enhances cell permeability and generates cavitations. Among other things, this technique can be used to extract anthocyanins and antioxidants. By inducing acoustic cavitation with ultrasound, we can increase the permeability of cell walls and the contact between the solvent and the sample. As well as changing the physical and chemical properties of materials, ultrasound degrades plant cell walls, which releases phytochemicals. It can extract phytochemicals on a small scale as well as a large one.  **Advantage:** shorter extraction and solvent use, Excellent at recovering. **Disadvantage:** High energy use can damage phytochemicals by creating free radicals.

2.6 Phytochemical screening

Phytochemicals (from the Greek phyton, meaning “plant”) are a group of naturally occurring compounds that may have either positive or negative impacts on human health. For a wide variety of medical conditions, people use medicinal plants, which are rich in a variety of phytochemicals. A plant is termed ‘medicinal’ due to its phytochemical components such as alkaloids, flavonoids, phenolics, tannins, saponins, steroids, glycosides, terpenes, and many more.

There is a large range of phytochemicals present in nature, with phenolics making up 45%, terpenoids and steroids 27%, and alkaloids 18%. While traditional medicine made use of whole plants for treatment, contemporary science has come a long way in discovering and extracting active components, which can subsequently be synthesized. These phytoconstituents can be utilized as building blocks for newly synthesized drugs due to the chemical structures they offer. The phytoconstituents of a plant can be a good indicator of its potential pharmacological action.

2.7 Instrumentation

2.7.1 Gas chromatography (GC)

Gas chromatography (GC) is an analytical technique that can be used with gas, liquid, and solid samples (heat-vaporized components). When a combination of compounds gets assessed with a GC system, each constituent may be detected and quantified. In the sample injection device, when the combined sample is injected into a GC system. In a GC system, the mobile phase, known as the carrier gas, moves successively from the sample introduction device to the

column and finally to the detector. A carrier gas transports target components evaporated in the sample injection device to the column. Once the combination of compounds penetrates the column, it gets separated into various components. A detector determines the amount of each component present. The detector converts the quantity of each component into electrical signals, which are subsequently sent to data processing. The data acquired can be used to determine the chemicals present in the sample and their amounts. A pictorial representation of the gas chromatography process is given in Figure 2.9 & its work is shown in Figure 2.10.

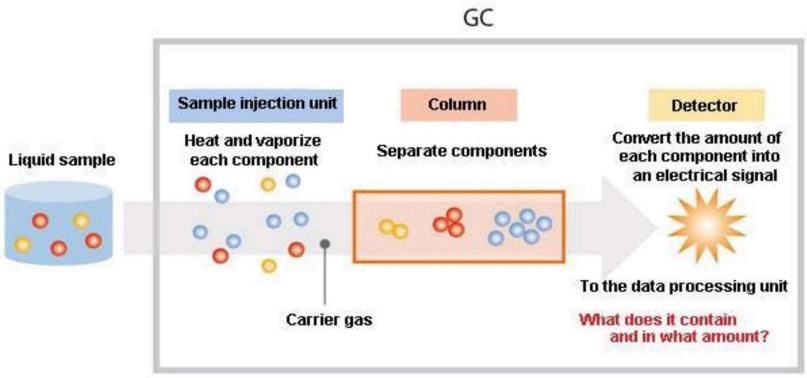


Figure 2.9 Gas Chromatography (GC) Model

The separation process takes place inside the column. Both the mobile phase and the chemically rich sample are loaded into the column at the same time. It is useful, it is often used as a carrier gas. Although both the mobile phase and the sample pass through the column, the chemicals control their relative velocity. Thus, not all chemicals reach the exit of the column at the same time. The compounds are then separated in this way. The vertical axis of the chromatogram indicates the electrical signals generated by the GC detector, and the horizontal axis indicates the time from sample injection. As a result, a series of peaks occur. As the components pass down the column, the gaseous mobile phase separates and adsorbs them into the solid or liquid phase.

The horizontal axis depicts the time it takes for the component to reach the detector. The vertical axis represents signal strength. The baseline is the area where no component is detected, while the peak corresponds to when the component is discovered. The retention time is the time required for spikes to appear after a sample is introduced into the system. Each component may be separated and identified since their elution times differ.

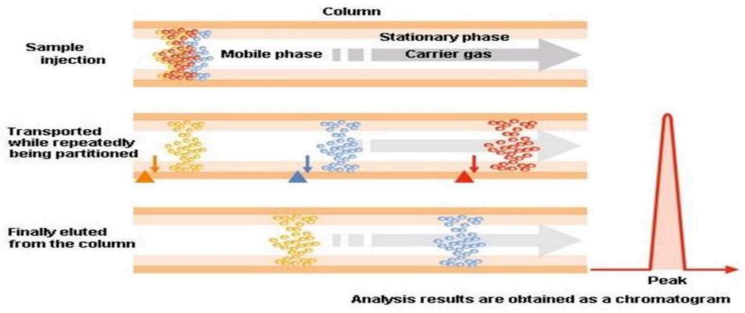


Figure 2.10 Working of GC

2.7.2 Gas chromatograph-mass spectrometry (GCMS)

Ions in the gaseous phase can be created, separated, and identified using the extremely sensitive detection technology called mass spectrometry (MS). When linked to a gas chromatograph, the eluted gases sort the ions in a vacuum according to their mass-to-charge ratios (m/z) and then calculate the intensity of each bond. Utilizing the recorded intensities, a sequence of mass spectra is produced to illustrate the relative ion strengths in relation to m/z. A mass chromatogram is produced after the GCMS run. Total Ion Current Chromatograms (TICs) are the chromatograms that are produced when the intensities of all the mass spectral peaks in one scan are added together. While competing GC detectors do exist, MS distinguishes themselves by offering quantitative and qualitative analysis. More than just a multi-component sample analyzer, GCMS can identify and quantify previously unseen compounds, as well as complex matrices.

A spectral library search can make use of data retrieved by GCMS, which includes retention time, molecular weight, and mass spectra. More accurate mass calculations and chemical composition estimates are possible with GCMS when supplemental software is used. For qualitative analysis, which requires the unique identification of molecules, this is crucial. The GC-MS analysis is shown in Figure 2.11.

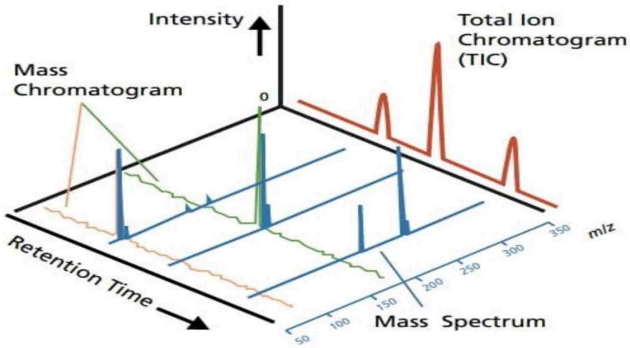


Figure 2.11 Gas chromatograph-mass spectrometry (GCMS)

Chapter III METHODS AND MATERIALS

Plant Collection and Identification

The leaves of *T. occidentalis* were meticulously collected from the local area of Delhi on December 10, 2023. The botanical sample underwent identification under the expert supervision of Prof. Vidhu Aeri, Pharmacognosy & Phytochemistry, School of Pharmaceutical Education and Research (SPER) at Jamia Hamdard, New Delhi. The sample was diligently preserved within the Department of Pharmacognosy &Phytochemistry, SPER Jamia Hamdard for comprehensive record-keeping.

3.1 Method Used

The Clevenger Method, also known as hydrodistillation, will be utilized in this research study to conduct the extraction of essential oil (Thakur, 2016). A combination of distilled water and glycerine is used to extract essential oil from Thuja leaves.

Procedure:

1. Firstly the leaves of *T. occidentalis* were plucked out of their stem then for extraction, a total of 200 grams of sample was split up into three separate batches. Stems and leaves of *T. occidentalis* are shown in Figures 3.1 & 3.2, and different batches of leaves are collected as can be seen in Figure 3.4.

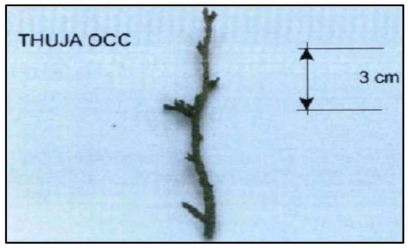


Figure 3.1 Stem of *T. occidentalis*

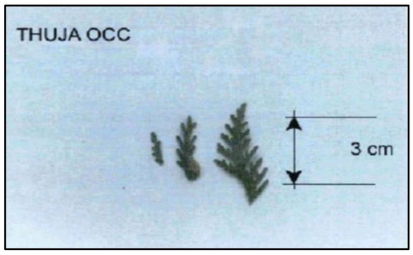


Figure 3.2 Leaves of *T. occidentalis*

2. In the first batch, roughly 95 grams of the material were weighed and then placed inside a round-bottom flask that contained 750 millilitres of distilled water. Figures 3.3 &3.3(a) describe the during-boiling and after-boiling effects on leaves.

Date	December 11, 2023
Temperature	40°C - 50°C
Time of Commencement	1:55 PM
Ebullition	2:45 PM
Time of Completion	4:45 PM

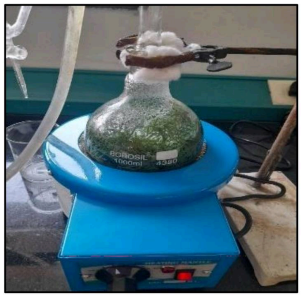


Figure 3.3 Boiling starts

Figure 3.3 (a)Dried Leaves

3. Now similarly in the second batch we took about 80 grams of sample in 750 ml of distilled water

Date	December 12, 2023
Temperature	60°C-100°C (Max-100°C)
Time of Commencement	10:20 AM
Ebullition	11:00 AM
Time of Completion	3:30 PM

4. After each interval of time, we collected the separated oil from the given mixture of water and steamed oil.
5. In the end, we were left with the isolated oil from the leaves of *T occidentalis* as shown in Figure 3.5.

Figure 3.4 Collection of leaves

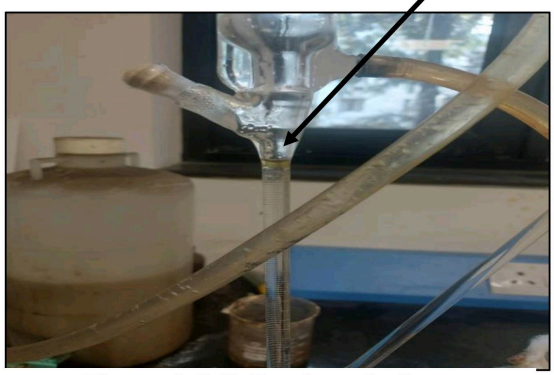


Figure 3.5 Collection of oil

3.2 Physiological Analysis

3.2.1 Moisture content

The test for moisture content determines both water and volatile substances. Drying can be

carried out either by heating at 100-105 °C or in a desiccator over phosphorus pentoxide R under atmospheric or reduced pressure at room temperature for a specified time (Joshi Arun B, 2014).

Procedure- In a pre-dried and weighed petri plate, 4 grams of the air-dried powdered sample was recorded as (W_b). To ensure that two subsequent weighings (W_c) do not differ by more than 5mg, the sample was air-dried in a hot air oven whose temperature was set at 100°C.

3.2.2 Total Ash Value

Finding out how much material is left over after ignition is the goal of the total ash method. Two kinds of ash exist: The “physiological ash,” which is directly produced by plants, & the “non-physiological” ash, which is the byproduct of any other substances that may have been adhered to the surface of the plant, including sand or mud. The test for total Ash value can be carried out in a muffle furnace at around 300-450°C for approx. 4 to 5 hrs, then the powdered sample needs to be weighed to calculate the total ash value.

Procedure- (W_1) was the weight of a desiccator-heated and cooled silicon crucible that had been cleaned. Carefully added to the silica crucible was the 2g (W_2) air-dried sample. The sample was put troughed to a series of controlled temperatures in an electrical muffle furnace until it turned white, confirming the absence of carbon. The temperature was around 450°C for approx. 3.5hrs. Using the above equation, the total ash content was determined after desiccation and reweighing (W_3).

3.3 Crude extract preparation

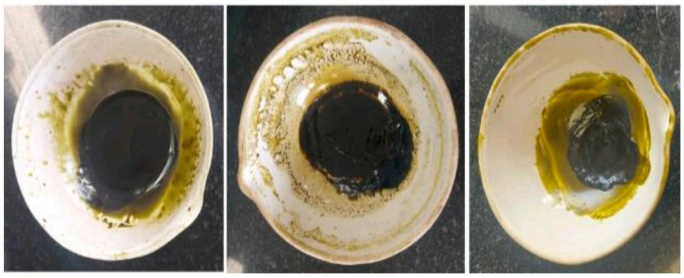
Method Used- Maceration

Procedure:

1. Pt. ether, Chloroform, and Methanol were used separately as a solvent medium for crude extract preparation.40 grams of sample was weighed separately for each solvent.
2. 500 mL of solvent was poured into the beaker containing the sample for every batch. The beakers were covered and kept undisturbed for 5 days

3. Filtration was carried out using Whatman filter paper No. 1. The filtrate was then kept in the water bath for approximately 30 minutes. The solvent was evaporated and crude extract was obtained on cooling.

After filtration



Dried extracts of $CHCl_3$, Pt. ether, CH_3OH

4. Filtration was carried out using Whatman filter paper No. 1. The filtrate was then kept in the water bath for approximately 30 minutes. The solvent was evaporated and crude extract was obtained on cooling

3.4 Phytochemical screening

3.4.1 Screening for Phytochemicals and Reagent Preparations

Screening for Alkaloid

1) Hager's test

1. Hager's reagent consists of concentrated picric acid in water.
2. Then combine the prepared reagent with a small quantity of plant extract (around 1-2 mL).
3. A creamy white precipitate will be apparent.

2) Mayer's test

1. Solution A, the base for Mayer's Reagent, consists of 60 mL of purified water and 1.3588 grams of mercuric chloride.
2. Mix 5 grams of potassium iodide with 10 ml of distilled water to make the solution B.
3. Prepare a solution by combining Solutions A and B. Pour in enough distilled water to make the final capacity 100 mL
4. Add a few mL of plant extract to the test tube together with one or two drops of Mayer's reagent.
5. A precipitate that is creamy white or yellow occurs in the presence of alkaloids.

Screening for Carbohydrates

1) Barfoed's test

Preparation of Barfoed's reagent:

1. Measure 30.5gm copper acetate and add 1.8mL glacial acetic acid.
2. Procedure: Take 1mL of filtrate and add 1mL of Barfoed's reagent. Heat it for 2 minutes.
3. Observation: A red precipitate will be formed. It indicates the presence of monosaccharides.

Screening for Reducing sugars

1) Fehling's test

1. Procedure: Add 1mL each of Fehling's solution A & B into 1mL of filtrate. Boil it in a water bath.
2. Observation: A red precipitate will be observed.

Screening for Flavonoids

1) Ferric chloride test

1. Procedure: Add a few drops of 10% ferric chloride solution in an aqueous solution of the extract.
2. Observation: A green precipitate will be observed.

2) Conc.H_2SO_4 test

1. Procedure: Add 1-2 mL of conc. H_2SO_4 directly into the plant extract.
2. Observation: An orange colour layer will be observed.

Screening for Glycosides

1) Keller-Killani test

1. Method: Dilute 1 mL of filtrate with 1.5 mL of glacial acetic acid, and then add 1 drop of 5% ferric chloride. Pour concentrated sulfuric acid along the sides of the test tube.
2. Observation: The acetic acid layer will produce a blue-coloured solution.

Screening for Phenolic compounds

1) Ferric chloride test

1. Procedure: Add a few drops of 5% ferric chloride solution in aqueous solution of the extract.
2. Observation: Dark green/bluish-black colour will be observed.

2) Test for Cartenoids:

1. Procedure: Take 1gm extract and add 10mL chloroform. Shake vigorously and filter. Add 2-3 mL of conc. H_2SO_4 into the filtrate.
2. Observation: A blue colour at the interface will be seen.

Screening for Tannins

1) Braymer's test

1. Procedure: Take 1mL filtrate and add 3mL distilled water. Add 3 drops of 10% ferric chloride solution into it.
2. Observation: A blue-green colour will be observed in the solution.

2) NaOH test

1. Procedure: Add 4mL of 10% NaOH in 0.4mL of plant extract. Shake well.
2. Observation: Formation of emulsion will be observed. It indicates hydrolysable tannins.

Screening for Phytosterols

1) Salkowski's test

1. Procedure: Add a few drops of conc. H_2SO_4 into the filtrate. Shake well and allow it to stand.
2. Observation: Red colour formation in the lower layer of the solution.

2) Hesse's response

1. Procedure: In 5mL of aqueous plant extract add 2mL of chloroform and 2mL of conc. H_2SO_4.
2. Observation: A pink/red ring will be formed in the lower chloroform layer.

Screening for Terpenoids

1) Standard test

1. Procedure: Add 2ml of chloroform in 5mL of plant extract. Add 3mL of conc. sulphuric acid. Boil it in the water bath.
2. Observation: A grey-coloured solution will be observed.

3.5 Quantification of Phytochemicals

3.5.1 Total Flavonoid Content (TFC)

We used an aluminium chloride colorimetric method to find out how many flavonoids were in the methanolic *T. occidentalis* extract. To measure the TFC of the extracts, rutin equivalents were used (mg of RE/g of extract).

Chemicals Required: Methanolic extract, standard rutin, 10% aluminium chloride, and 1 M sodium acetate.

Preparation of Standard Rutin solution -A rutin stock solution was produced using methanol, at a concentration of 1000 µg/mL. Concentrations of 20, 40, 60, 80, 100, and120 µg/mL were obtained by further diluting the stock solution with methanol.

Preparation of Test Solution -Methanol was used to generate a test solution with a concentration of 1000 µg/mL. The concentration of 20µg/mL was achieved by further diluting the stock solution with methanol.

Preparation of Blank solution- In a test tube, 2.8 mL of pure water, 2 mL of methanol, 0.1 mL of 1 M sodium acetate, and 1 mL of 10% aluminium chloride were added.

Preparation of Standard curve- In the test tube, 1.5 mL of methanol and 2.8 mL of distilled water were incorporated with 0.5 mL of rutin dilutions (20 to 120 µg/mL), 0.1 mL of 10% aluminium chloride, and 0.1 mL of 1 M sodium acetate. Using a blank sample as a reference, each dilution was incubated for 30 minutes at room temperature in the dark before being evaluated for absorbance at 415 nm using a UV/VIS Spectrophotometer. Absorbance (Y-axis) was plotted against concentration (X-axis) to construct the standard curve.

The same amounts of distilled water (2.8 mL), methanol (1.5 mL) and sodium acetate (1 M) and 10% aluminium chloride (0.1 mL each) were subsequently added to the test solution (0.5 mL). A blank sample was used as a reference, and after 30 minutes of incubation in the dark at room temperature, the test sample was tested for absorbance at 415 nm using a UV/VIS Spectrophotometer. The total flavonoid content was calculated as rutin equivalent (RUE) using the following equation:

$$TFC = C \times V \times D \times 100 / M$$

The following factors determine the total flavonoid concentration (TFC) in milligrams per gram of extract (RUE): M is the mass of the extract (g), V is its volume in (mL), and D is its dilution factor. With the use of the calibration curve, we can find the rutin concentration, C (µg/mL).

3.5.2 Total Phenolic Content (TPC)

When analysing the methanolic *T. occidentalis* extract for total phenolic content, we turned to the Folin-Ciocalteu method. We looked at milligrams of gallic acid per gram of extract to determine the TPC of the extracts.

Chemicals Required: Included in the mixture are methanolic extract, standard gallic acid, 7.5 per cent (w/v) sodium carbonate, and 10% Folin-Ciocalteu reagent.

Preparation of Standard Gallic acid solution- A 1000 µg/mL concentration of gallic acid was used to make the methanol stock solution. More methanol was added to the stock solution to acquire the concentrations of 20, 40, 60, 80, 100, and 120 µg/mL.

Preparation of Test solution methanol-based test solution was prepared with a concentration of 1000 µg/mL. The stock solution was further diluted with methanol to obtain concentrations of 20µg/mL.

Preparation of Blank solution test tube was filled with two mL of 10% F-C reagent, four mL of 7.5% sodium carbonate, half an mL of methanol, and ten mL of distilled water.

Preparation of Standard Curve -Each of the two mL of gallic acid dilutions (20 to 120 µg/mL) in the test tube was supplemented with two mL of 10% F-C reagent and four mL of 7.5% sodium carbonate. We compared the absorbance at 765 nm of each dilution to a blank sample after incubating them in the dark at room temperature for 2 hours. The standard curve was created by plotting absorbance (Y-axis) against concentration (X-axis).In an additional test tube, 0.5 mL of test solution, 2 mL of 10% F-C reagent, and 4 mL of 7.5% sodium carbonate were mixed according to the same protocol. After a two-hour dark room temperature incubation period, the absorbance at 765 nm was measured for each dilution using a UV/VIS

Spectrophotometer, in reference to a blank sample. The total phenolic content was determined using a gallic acid equivalent (GAE) measurement:

$$TPC = C \times V \times D \times 100 / M$$

TPC is the total phenolic content in mg/g of the extracts as GAE, C is the concentration of gallic acid established from the calibration curve µg/mL, V is the volume of the extract solution in mL, D is the dilution factor and M is the weight of the extract in g.

3.6 MIC Assay

The Minimum Inhibitory Concentration (MIC) of *Bacillus cereus* was assessed by utilising methanol, chloroform, and petroleum ether extracts derived from *T. occidentalis*, whereas PHMB was employed as a reference antimicrobial agent. The efficacy of extracts determines their antibacterial effects on certain pathogens.

3.7 ZOI assay

The zone of inhibition (ZOI) for *Bacillus cereus* was assessed using different concentrations of chloroform, methanol, and petroleum ether extracts. Streptomycin was used as a positive control, and MQ water as a negative control over Mueller Hinton Agar (MHA) culture plates.

3.8 Molecular docking

Utilizing the Schrödinger molecular modelling software, molecular docking studies were conducted to gain insight into the interaction between certain ligands and proteins from *Bacillus cereus* (PDB IDs 7NMQ and 5FQB). The interactions between the ligands and proteins offer valuable insights into the molecular mechanisms that underlie the antibacterial activity of these extracts.

Chapter – IV RESULT AND DISCUSSION

4.1 Amount of Essential oil extracted

From a 200-gram sample of *T. occidentalis* dried leaves in 1075 mL of distilled water we extracted approx. 0.8 mL of Essential oil (EO).

4.2 Physiological Analysis

4.2.1 Moisture content

Moisture contained as the rate of time concerning the weight of the sample is expressed in Table 4.1 and Loss on drying is depicted in Figure 4.1.

- Weight of the empty pre-dried Petri dish (W_a): 51.34 g.
- Weight of the sample taken: 4 g.
- Weight of the Petri dish and sample (W_b): 55.34 g.
- Temperature recorded: 100-105°C.

Table 4.1 Data description of Moisture contain

Sno.	Time (mins)	Weight of the sample (g)	Loss of weight (g)
01.	0	4.00	-
02.	15	3.936	0.064
03.	30	3.857	0.079
04.	45	3.787	0.070
05.	60	3.715	0.072
06.	75	3.643	0.072

Dried Weight (W_c) = 54.983 g.

$$\text{Moisture (\%)} = \frac{(W_b - W_c) * 100}{(W_b - W_a)}$$

$$= [(55.34-54.983)/(55.34-51.34)] * 100$$

$$= 8.925\ \%.$$

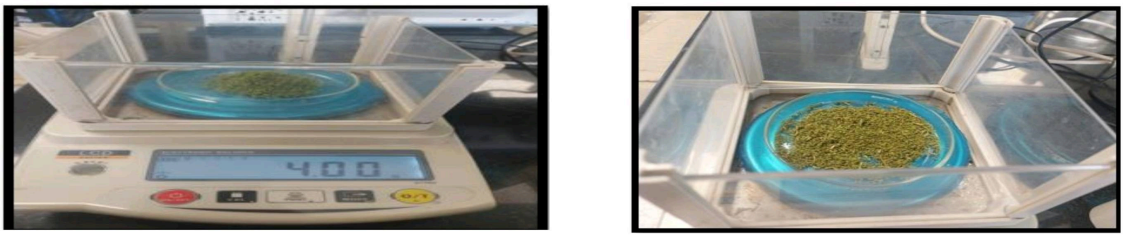


Figure 4.1 Loss on Drying of Thuja leaves

4.2.2 Total Ash Value

Weight of the preheated empty crucible (W_1) = 22.834 g.

Weight of the sample taken = 2.012 g.

Weight of the crucible after addition of sample (W_2) = 24.846 g.

Weight of the crucible after formation of ash (W_3) = 22.932 g.

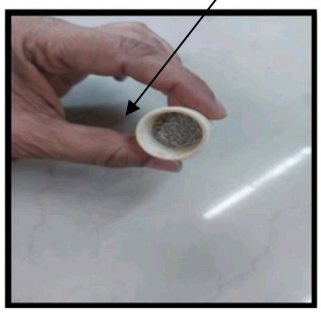
Total Ash (%) = $[(W_3 - W_1)]/ [(W_2 - W_1)] * 100$

= $[(22.932 - 22.834)]/ [(24.846 - 22.834)] * 100$

= 4.87%.

Weighing of that ash

White powdered ash



4.3 Results for Phytochemical Screening

Table 4.2 Phytochemical screening of *T. occidentalis* leaf extracts

S. No.	Tests for Phytochemicals	Petroleum Ether	Chloroform	Methanol
(A)	**Alkaloids** 1) Hager's test 2) Mayer's test	 – –	 – –	 + +
(B)	**Carbohydrates** 1) Barfoed's test	 –	 –	 –
(C)	**Reducing sugars** 1) Fehling's test	 –	 +	 +
(D)	**Flavonoids** 1) Ferric chloride test 2) Conc.H_2SO_4 test	 – +	 – +	 + +
(E)	**Glycosides** 1) Keller Kiliani test	 +	 +	 –
(F)	**Phenolic Compounds**			

	1) Ferric chloride test 2) Cartenoids test	− +	± +	+ +
(G)	**Tannins** 1) Braymer's test 2) NaOH test	 − −	 − ±	 + +
(H)	**Phytosterols** 1) Salkowski's test 2)Hesse's response	 − −	 − −	 + +
(I)	**Terpenoids** 1) Standard Test	 +	 +	 +

Note:
(+): Presence of the phytochemical.
(−): Absence of the phytochemical.
(±): Slight presence of the phytochemical.

4.4 TFC Analysis

Absorbance at different concentrations of Rutin are discussed in Table 4.3, and Figure 4.2 and data descriptions for test solution are discussed in Table 4.4.

Table 4.3 Data of conc. Of Rutin and absorbance

Conc. Of Rutin(µg/mL)	Absorbance(415 nm)
20	0.346
40	0.417
60	0.512
80	0.608
100	0.715
120	0.858

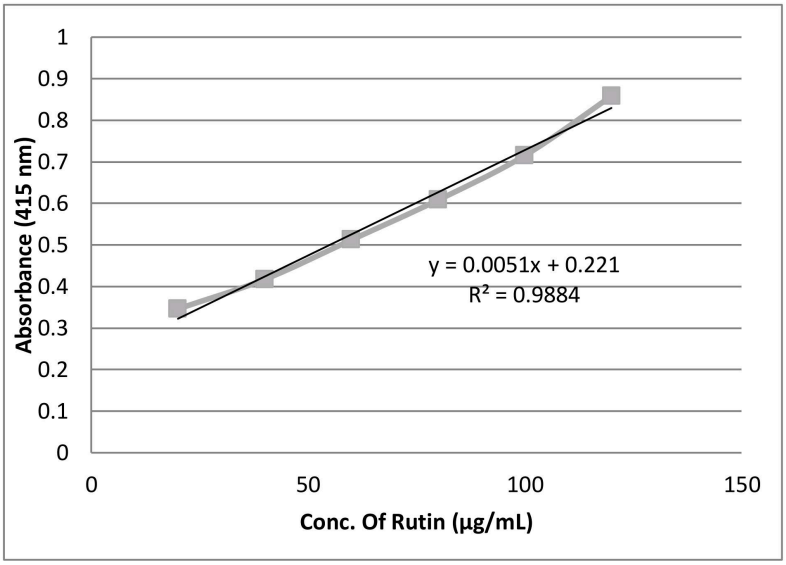


Figure 4.2 Calibration Curve of Rutin

Table 4.4 Data description of Test solution

Sample Soln. (µg/ml)	Weight of Extract (g)	Absorbance (nm)	Rutin Conc. (µg/ml)	Rutin Conc. (mg/ml) (C)	Dilution Factor (D)	TFC =C×V×D×100/M (mg/g)
20	20×10^{-6}	0.879	129.01	0.129	50	32.25

4.5 TPC Analysis

Absorbance at different concentrations of GA is discussed in Table 4.5 and Figure4.3 and data description for the test solution are discussed in Table 4.6

Table 4.5 Data of conc. Of GA and absorbance

Conc. Of GA (µg/mL)	Absorbance (765 nm)
20	0.141
40	0.237
60	0.358
80	0.474
100	0.608
120	0.796

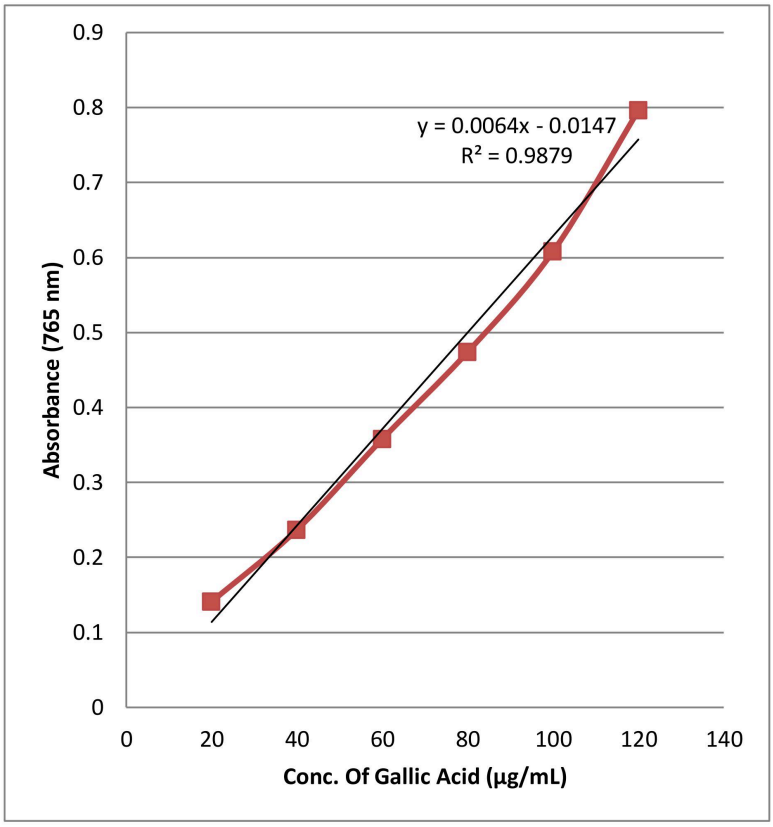


Figure 4.3 Calibration Curve of Gallic Acid

Table 4.6 Data description of Test solution

Sample Soln (µg/ml)	Weight of Extract (g)	Absorbance (nm)	GAE Conc. (µg/ml)	GAE Conc. (mg/ml)(C)	Dilution Factor (D)	TPC=C×V×D×100/M (mg/g)
20	20×10^{-6}	0.268	47	0.047	50	11.75

4.6 Instrument specifications

GC Model: Agilent 7890A with 7683B Autosampler.

GCMS Model: Perkin Elmer Clarus 500 with a mass spectrophotometer.

4.6.1 Analysis- The comparative analysis of the compounds present in the three different extracts (Chloroform, Petroleum Ether, and Methanolic) from Tables 4.7, 4.9 and 4.11 and Figures 4.4 to 4.9 provides insights into the chemical diversity and abundance within each solvent extract. The tables list compounds along with their molecular weight (M.W.), gas chromatography retention time (GCRT), gas chromatography-mass spectrometry retention time (GCMS RT), and the percentage area (% Area) that indicates the relative concentration of each compound**.**

Chloroform extract:

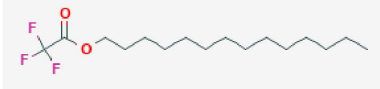
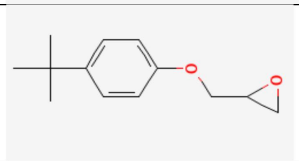
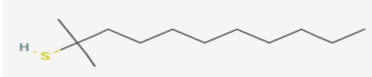
Table 4.7 Compounds present in Chloroform extract

S. No	Compound	M.W.	GCRT (min)	GCMS RT(min)	% Area
1)	α-Pinene (R)	136	28.8	27.3	3.92
2)	3-Dodecene	168	29.2	27.7	5.19
3)	3-Carene	136	30.4	28.7	2.81
4)	2-Methylundecane-2-thiol	202	33.9	31.9	22.58
5)	Bicyclo[2.2.2]octane-1,4-diol	142	34.2	32.1	2.75
6)	trans-p-Menth-2-enol	152	34.3	32.3	2.38
7)	Caren-4-ol	152	34.9	32.7	3.43
8)	Tetradecyl trifluoroacetate	310	40.6	37.7	26.76
9)	p-tert-Butylphenyl glycidyl ether	206	46.5	42.6	30.16

Interpretation:

The chloroform extract consists of nine compounds with varying molecular weights and retention times. The major compounds by % Area are mentioned in Table 4.8.

Table 4.8 Major Compounds present in Chloroform extract

	COMPOUND	% Area	STRUCTURE
1)	Tetradecyl trifluoroacetate	(26.76%)	
2)	p-tert-Butylphenylglycidyl ether	(30.16%)	
3)	2-Methylundecane-2-thiol	(22.58%)	

These three compounds dominate the chloroform extract, making up the majority of the content. The presence of α-Pinene (R), 3-Dodecene, and Caren-4-ol in smaller amounts indicate a diverse profile but with significant contributions from the higher conc. compounds.

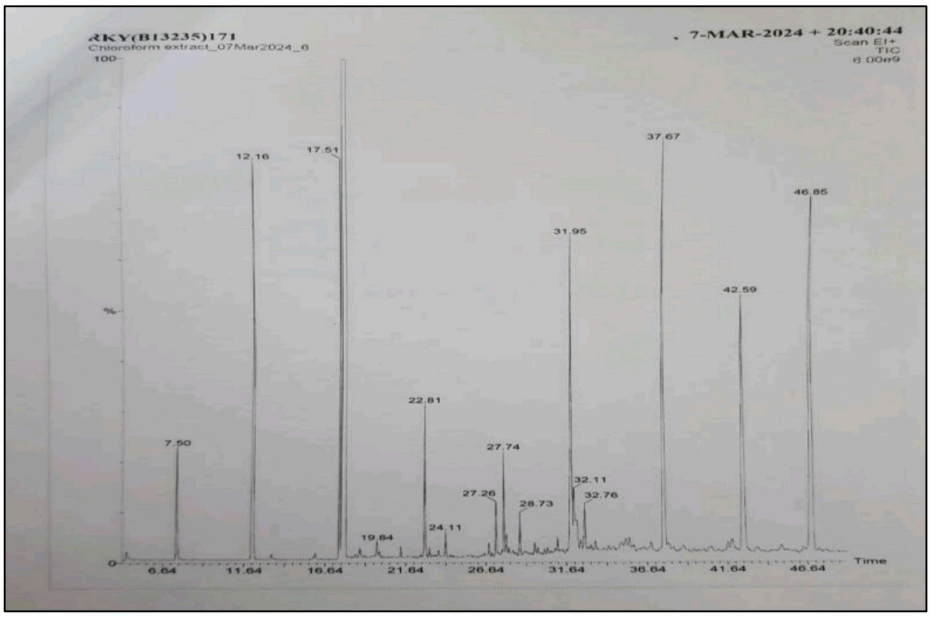


Figure 4.4 GCMS of Chloroform extract

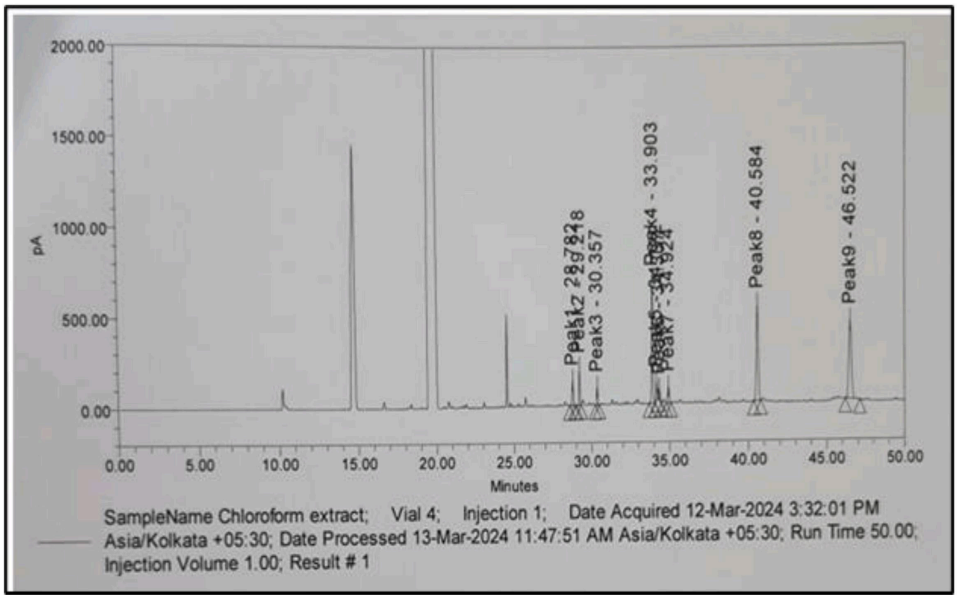


Figure 4.5 GC of Chloroform extract

Petroleum Ether extract:

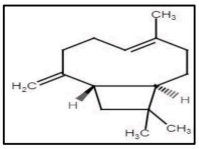
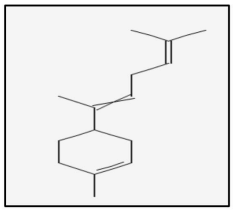
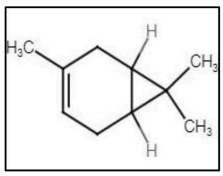
Table 4.9 Compounds present in Petroleum Ether extract

S.No.	Compound	M.W.	GCRT (min)	GCMS RT (min)	% Area
1)	α-Pinene (R)	136	28.8	27.3	10.53
2)	3-Carene	136	30.4	28.7	16.45
3)	Thymol	150	34.2	32.1	4.99
4)	Caren-4-ol	152	34.4	32.3	8.33
5)	γ-Terpineol	154	39.7	36.9	4.26
6)	Caryophyllene	204	45.7	41.9	34.54
7)	α-Bisabolene	204	47.5	43.4	20.9

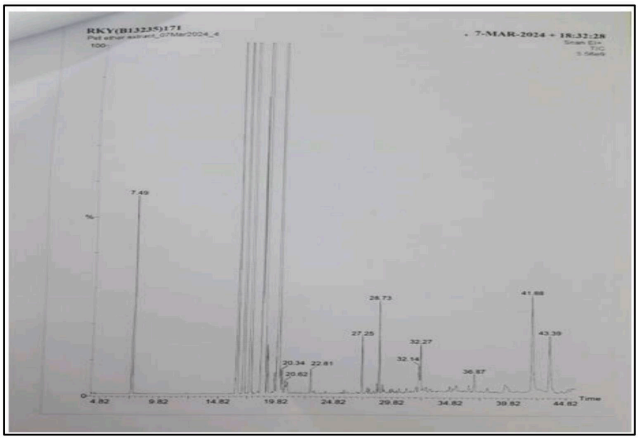
Interpretations:

In the petroleum ether extract, seven compounds were identified. The most prominent compounds by % Area are depicted in Table 4.10.

Table 4.10 Major Compounds present in Petroleum Ether extract

S.No.	COMPOUND	% Area	STRUCTURE
1)	Caryophyllene	(34.54%)	
2)	α-Bisabolene	(20.9%)	
3)	3-Carene	(16.45%)	

Caryophyllene and α-Bisabolene together comprise over half of the petroleum ether extract. The higher concentration of these sesquiterpenes suggests that petroleum ether is particularly effective at extracting these types of compounds. Other compounds like α-Pinene (R) and Caren-4-ol are also present but in lower concentrations.



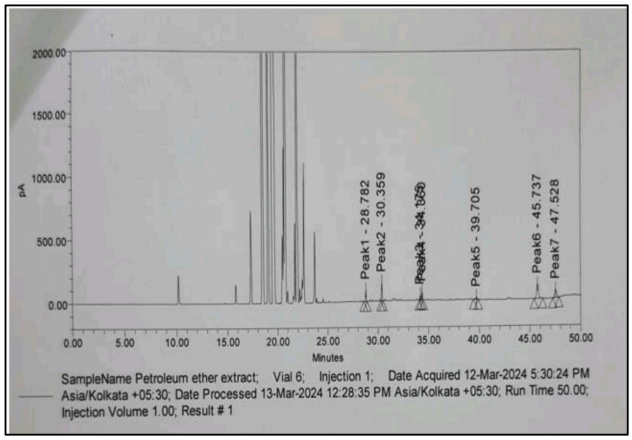


Figure 4.6 GCMS of Pt. Ether extract

Figure 4.7 GC of Pt. Ether extract

Methanolic extract:

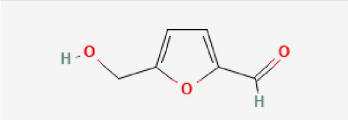
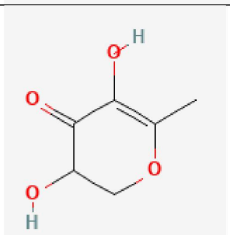
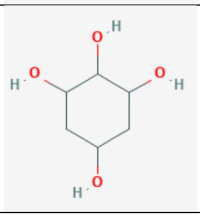
Table 4.11 Compounds present in Methanolic extract

S. No.	Compound	M.W.	GCRT (min)	GCMS RT (min)	% Area
1)	DL-Glyceraldehyde	180	26.4	24.8	6.1
2)	Diglycerol	166	30.7	26.4	5.61
3)	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	144	33.1	31.2	16.13
4)	5-Hydroxymethylfurfural	126	34.1	32.1	16.76
5)	Acetin	134	34.2	32.5	7.67
6)	Caryophyllene	204	41.3	41.9	5.02
7)	Santolina Triene	136	44.2	43.4	6.79
8)	Methyl β-D-glucopyranoside	194	45.7	45.0	6.8
9)	1,2,3,5-Cyclohexanetetrol	148	47.6	46.0	12.3
10)	Sequoyitol	194	49.5	46.9	7.69

Interpretations:

The methanolic extract displays ten compounds, with notable contributions. This extract shows a diverse range of compounds, including various polyols and heterocyclic compounds. The presence of DL-Glyceraldehyde and Diglycerol also highlight. The most prominent compounds by % Area are depicted in Table 4.12.

Table 4.12 Major Compounds present in Methanolic extract

	COMPOUND	% Area	STRUCTURE
1)	5-Hydroxymethylfurfural	(16.76%)	
2)	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	(16.13%)	
3)	1,2,3,5-Cyclohexanetetrol	(12.3%)	

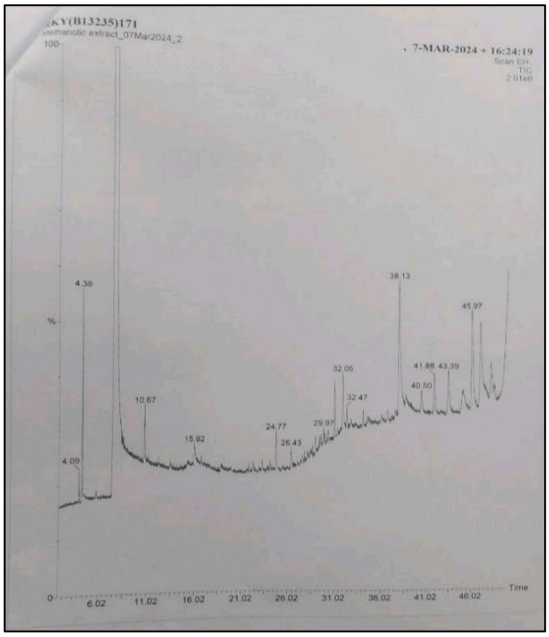


Figure 4.8 GCMS of Methanolic Extract

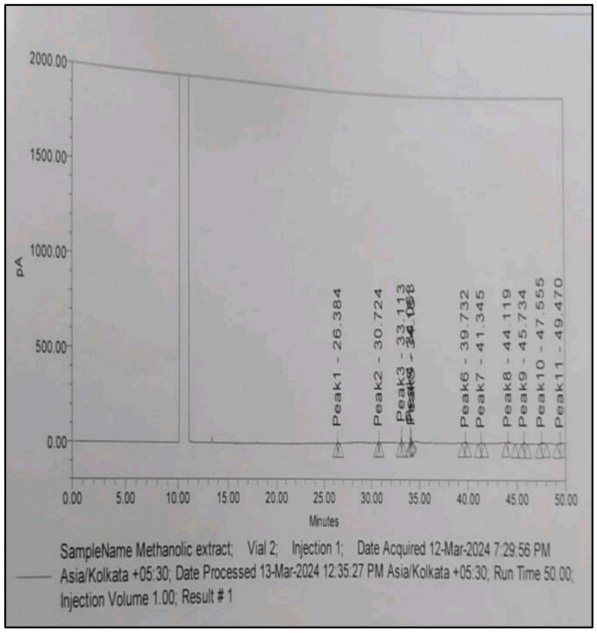


Figure 4.9 GC of Methanolic Extract

4.6.2 Comparative Analysis

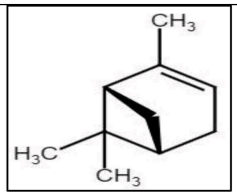
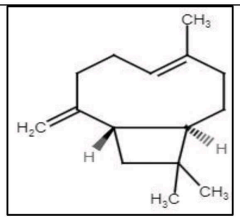
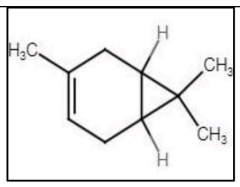
The methanolic extract contains the highest number of unique compounds, suggesting that methanol is more effective for the extraction of a wide range of compounds. In contrast, the chloroform and petroleum ether extracts have fewer unique compounds indicating these solvents may be more selective in their extraction capabilities.

α-Pinene (R) and 3-Carene are present in both the chloroform and petroleum ether extracts, but not in the methanolic extract. Caryophyllene appears in both the petroleum ether and methanolic extracts, though in a much higher concentration in the petroleum ether extract. The retention times (both GCRT and GCMS RT) vary for the same compounds across different extracts, indicating the solvent's impact on the chromatography process.

The percentage area of compounds like p-tert-butylphenyl glycidyl ether (30.16%) in chloroform and Caryophyllene (34.54%) in petroleum ether indicates that these compounds are extracted more efficiently by their respective solvents. The methanolic extract shows a more balanced distribution with several compounds having significant percentage areas, indicating a more even extraction profile for various polar compounds.

Overall, chloroform is more effective for isolating large, less polar compounds. Petroleum ether favours the extraction of sesquiterpenes and other non-polar to moderately polar compounds. Methanol excels in extracting a wide variety of polar and semi-polar compounds. Understanding these differences allows for targeted extraction based on the desired compound profile, improving efficiency in chemical analysis and potential applications in pharmacology, environmental testing, and food safety. And the summary of common compounds observed in all three extracts with their observed biological activities are tabulated in Table 4.13.

Table 4.13 Common Compounds

Compound	Structure	Biological Activities
α-Pinene		1)Antibacterial 2)Anticoagulant 3)Antimalarial 4)Anti-inflammatory 5)Anticonvulsant
Caryophyllene		1)Antibacterial 2)Antioxidant 3)Gastroprotective 4)Anxiolytic 5)Anti-inflammatory
3-Carene		1)Antimicrobial 2)Antioxidant 3)Anticancer 4)Semiochemical 5)Fumigant properties

4.7 Analysis of Minimum Inhibitory Concentration (MIC) for *Bacillus cereus* (BC)

Optical density (OD) measurements were collected at 0 and 24 hours at various concentrations (300 µg/mL, 600 µg/mL, 900 µg/mL, and 1200 µg/mL) to evaluate the suppression of bacterial growth. The findings demonstrated that the methanol and chloroform extracts exhibited considerable inhibition of *Bacillus cereus*, with methanol displaying greater efficacy at lower doses. The efficacy of the petroleum ether extract was lower, as indicated by the greater rates of bacterial survival. PHMB demonstrated the highest level of antibacterial effectiveness, consistently obtaining 100% per cent of bacterial eradication at the highest concentration. The results indicate that extracts of *T. occidentalis* containing methanol and chloroform exhibit significant antibacterial activity, making them promising candidates for the development of antimicrobial drugs.

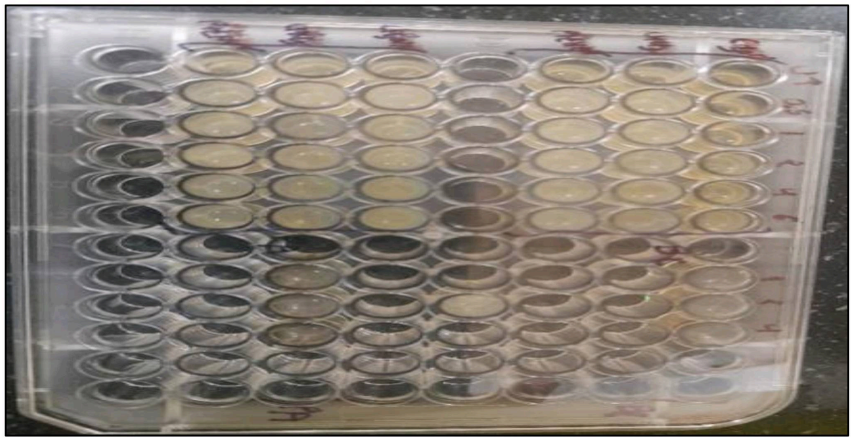


Figure 4.10 96 Well plate for MIC data

The above figure 4.10, of a 96-well plate, is commonly used in scientific research for determining minimum inhibitory concentrations (MIC) of antimicrobials. The plate consists of a rectangular grid of 96 individual wells. Each well can hold a small volume of liquid, allowing researchers to test multiple samples simultaneously. In MIC assays, different concentrations of antimicrobial agents are added to each well, along with a standardized number of microorganisms. The plate is then incubated, and researchers measure microbial growth in each well. The MIC is the lowest concentration of the antimicrobial agent that inhibits the visible growth of microorganisms.

4.7.1 Optical Density (OD) Measurements

The optical density (OD) measurements of *Bacillus cereus*, after exposure to various concentrations of the extracts and PHMB, were recorded at 0 hours and 24 hours. The discrepancy in optical density (OD) measurements between the 24-hr and 0-hr timepoints was utilized to evaluate the extent of bacterial proliferation and is displayed in Table 4.14.

Table 4.14 OD Difference (24 hr - 0 hr)

Conc. (µg/ml)	Methanol 1st batch	Methanol 2nd batch	Chloroform 1st batch	Chloroform 2nd batch	Pt. Ether 1st batch	Pt. Ether 2nd batch	PHMB 1stbatch	PHMB 2ndbatch
300	1.063	1.002	0.83	0.784	1.137	1.116	0.231	0.315
600	1.058	0.986	1.168	0.615	1.021	1.056	0.152	0.205
900	0.987	0.981	0.992	1.189	0.986	0.978	0.17	0.168
1200	0.887	0.919	1.088	0.878	0.966	1.008	0.047	0.006
Solvent	0.897	0.919	1.103	0.845	0.08	1.019	0.027	0.001

4.7.2 Extracts Survival Rate and Percentage Killing- The survival rates and percentage of killings for the extracts were determined by applying the formula (Extracts * 100 / Bavg). Table 4.15 and Figure 4.11 & Table 4.16 displays the Avg. survival rate for each extract and PHMB at various concentrations, while Table 4.17 and Figure 4.12 show the proportion of organisms killed.

Table 4.15 Extracts * 100/Bavg

Conc. (µg/ml)	Methanol 1st batch	Methanol 2nd batch	Chloroform 1st batch	Chloroform 2nd batch	Pt Ether 1st batch	Pt Ether 2ndbatch	PHMB 1st batch	PHMB 2ndbatch
300	98.42593	92.77778	76.85185	72.59259	105.2778	103.3333	21.38889	29.16667
600	97.96296	91.2963	108.1481	56.94444	94.53704	97.77778	14.07407	18.98148
900	91.38889	90.83333	91.85185	110.0926	91.2963	90.55556	15.74074	15.55556
1200	82.12963	85.09259	100.7407	81.2963	89.44444	93.33333	-	-
Solvent	83.05556	85.09259	102.1296	78.24074	7.407407	94.35185	-	-

Table 4.16 Average Survival Rate of extracts on BC

Conc. (µg/ml)	Methanol extract	Chloroform extract	Pt. ether extract	PHMB
300	95.60185	74.72222	104.3056	25.27778
600	94.62963	82.5463	96.15741	16.52778
900	91.1111	100.9722	90.92593	15.64815
1200	83.61111	91.01852	91.38889	-
Solvent	84.07407	90.18519	50.87963	-

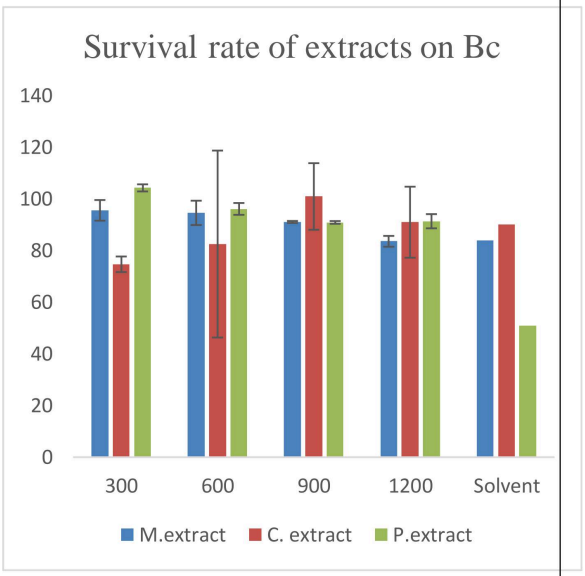


Figure 4.11 Average survival rate of BC

Table- 4.17 Average Survival Rate of extracts on BC

Conc.	M. extract	C. extract	P. extract	PHMB
300	4.398148	25.27778	-4.30556	74.72222
600	5.37037	17.4537	3.842593	83.47222
900	8.888889	-0.97222	9.074074	84.35185
1200	16.38889	8.981481	8.611111	100

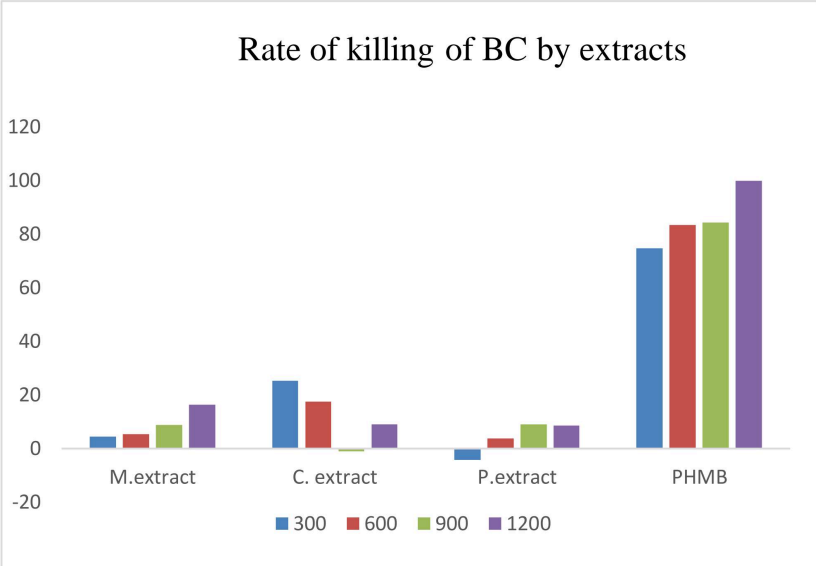


Figure 4.12 Percentage killing by extracts on BC

4.8 Analysis of Zone of Inhibition

Petri plates for all three extracts for zone analysis on BC is shown in Figure 4.13

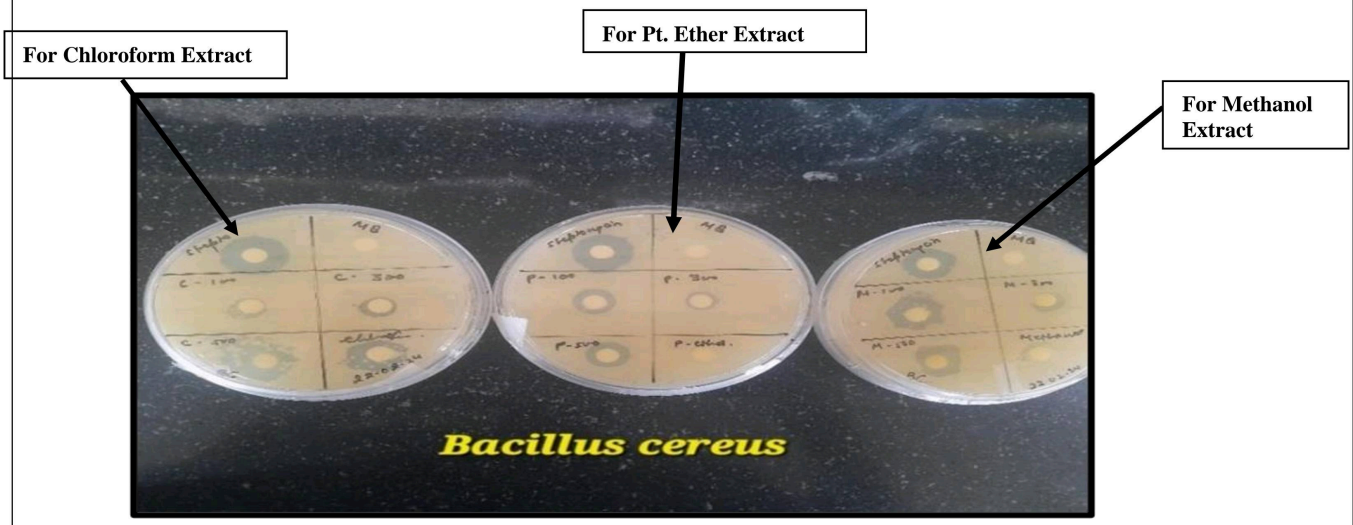


Figure 4.13 Petri plates for ZOI of all three extracts

4.9 Analysis of Zone of Inhibition (ZOI) for *Bacillus cereus*

The ZOI values are presented in Tables 4.18 to 4.20 and Figure 4.14 to 4.16

Table 4.18 ZOI of Chloroform Extract

Conc.	ZOI (mm)
c-100	10
c-300	10
c-500	18
chloroform	15
Streptomycin	17
MQ	0

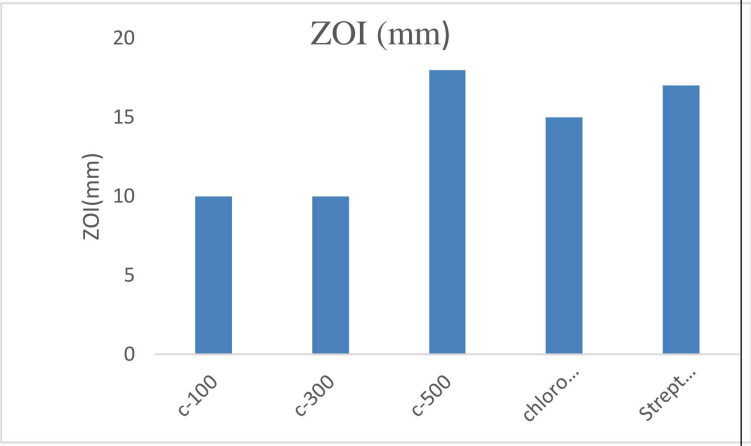


Figure 4.14 Graph of ZOI of Chloroform Extract

Table 4.19 ZOI of Methanol Extract

Conc.	ZOI (mm)
m-100	16
m-300	10
m-500	15
Methanol	11
Streptomycin	16
MQ	0

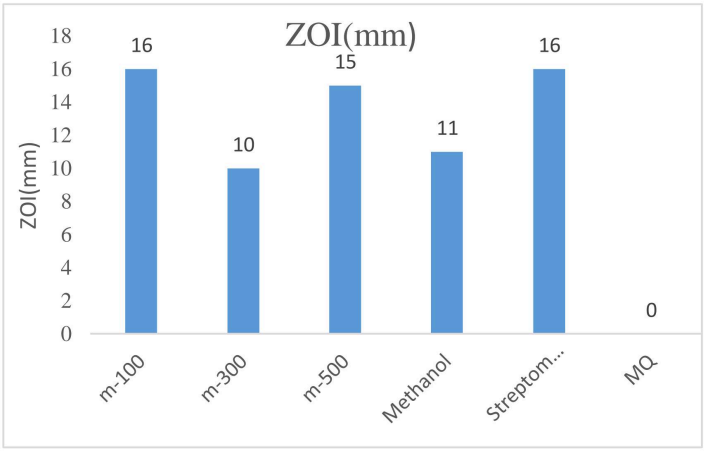


Figure 4.15 Graph of ZOI of Methanol Extract

Table 4.20 ZOI of Pt. ether Extract

Conc.	ZOI (mm)
p-100	12
p-300	10
p-500	12
Pt. Ether	0
Streptomycin	17
MQ	0

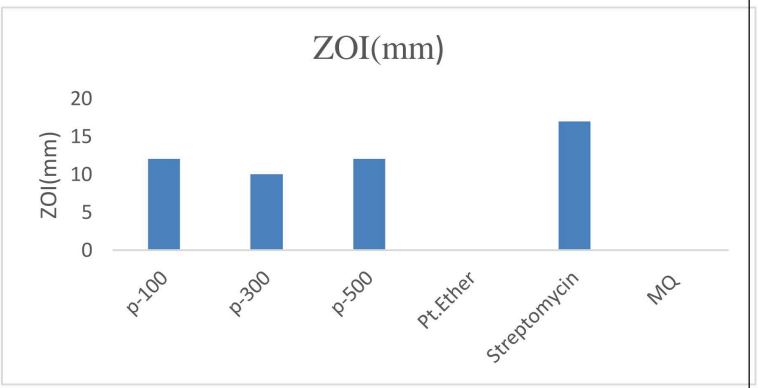


Figure 4.16 Graph of ZOI of Pt. ether Extract

4.9.1 Statistical Analysis

In Table 4.21, one can find the standard deviations for the extracts across all of the different concentrations. The reliability and reproducibility of the experimental results are both ensured by this statistical analysis.

Table 4.21 Standard Deviation

Std. Dev.	Methanol extract	Chloroform extract	Pt. Ether extract
300	3.993844	3.011751	1.37493
600	4.714045	36.20649	2.29155
900	0.392837	12.89815	0.523783
1200	2.095131	13.7493	2.74986
	1.440403	16.892	61.47901

4.10 Interpretation and Discussion

This section involved the assessment of the Minimum Inhibitory Concentration (MIC) for *Bacillus cereus* utilising several solvent extracts, namely methanol, chloroform, and petroleum ether. Additionally, PHMB was used as a standard antimicrobial agent. The evaluation included measurements of optical density (OD) at various time intervals (0 hours and 24 hours) and concentrations (300 µg/mL, 600 µg/mL, 900 µg/mL, and 1200 µg/mL). The OD measurements were utilised to ascertain the inhibition of bacterial growth by determining the disparity in OD values over a span of 24 hours.

The survival rates and lethality percentages of *Bacillus cereus* were determined for each extract and PHMB. The bactericidal activities of methanol and chloroform extracts exhibited variability, with methanol displaying greater inhibition at lower doses in comparison to chloroform. Petroleum ether exhibited the highest rate of survival, suggesting a lesser level of antibacterial effectiveness. PHMB demonstrated the highest level of antibacterial effectiveness, consistently obtaining a 100% rate of bacterial eradication at the maximum dose of 1200 µg/mL.

The zone of inhibition (ZOI) assays confirmed the growth inhibition results, demonstrating the antibacterial effectiveness of methanol and chloroform extracts. Petroleum ether displayed smaller inhibition zones. Streptomycin was used as a positive control to confirm the efficacy of the extracts.

MIC Result Analysis

The growth inhibition data demonstrate substantial antibacterial efficacy of the studied extracts against *Bacillus cereus*. Out of the extracts tested, petroleum ether had the highest proportion of survival, indicating a negative correlation with its antibacterial effectiveness. The bactericidal activities of methanol and chloroform extracts exhibited variability, with methanol displaying greater inhibition at lower doses in comparison to chloroform. PHMB demonstrated the highest level of antibacterial effectiveness, consistently achieving a 100% rate of bacterial eradication at a concentration of 1200 µg/mL. These findings demonstrate the potent ability of the substance to kill *Bacillus cereus* bacteria, suggesting its potential as an effective treatment for bacterial infections.

ZOI (Zone of Inhibition) Analysis

The zone of inhibition results confirm the growth inhibition data, demonstrating the efficacy of methanol and chloroform extracts at various doses. Petroleum ether, despite having a high proportion of survival in the MIC analysis, showed measurable inhibition zones, though smaller compared to methanol and chloroform extracts. Methanol extracts demonstrated significant inhibition zones even at lower concentrations, indicating strong antibacterial activity. Chloroform extracts showed moderate zones of inhibition, highlighting some level of effectiveness. PHMB exhibited the largest zones of inhibition, reinforcing its superior antibacterial properties observed in the MIC analysis.

Molecular Docking Analysis

The docking results demonstrated robust binding affinities between the ligands and the target proteins, confirming the antibacterial activity seen in the experimental experiments. The docking studies provide additional evidence, showing that the ligands had a high affinity for the selected *Bacillus cereus* evaluated proteins the selected *Bacillus cereus* evaluated proteins. Some of the docking stores are mentioned in Figure 4.17.

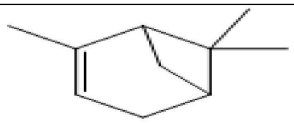
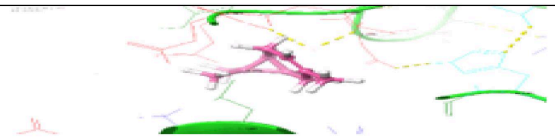
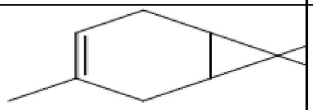
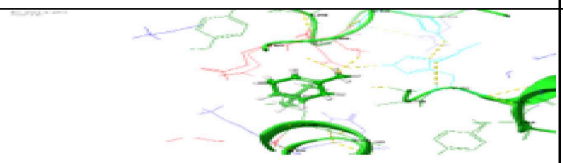
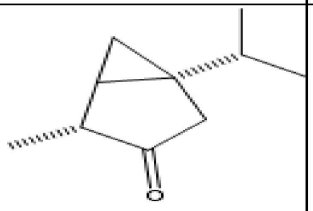
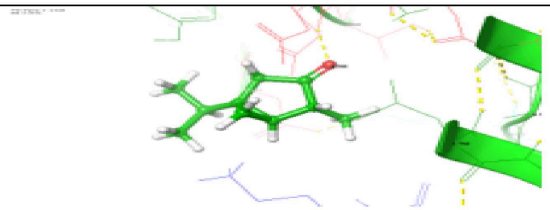
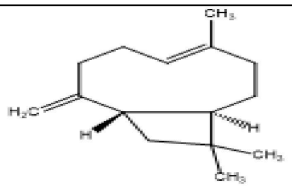
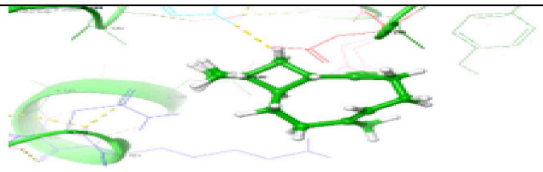
COMPOUND NAME	STRUCTURE	DOCKING IMAGES	DOCKING SCORE
α- PINENE			-4.319
3 CARENE			-4.839
THUJONE			-4.587
CARYOPHYLLENE			-4.615

Figure 4.17 Docking modules

Chapter – V CONCLUSION AND FUTURE RECOMMENDATIONS

5.1 Conclusion

The research analysis investigated the ability of different *T. occidentalis* extracts to inhibit the growth of *Bacillus cereus*. The study employed petroleum ether, chloroform, and methanol extracts and evaluated their effectiveness against PHMB, a common antibacterial agent. Petroleum extracts showed strong antibacterial activity, with methanol being more effective at lower concentrations, according to the Zone of Inhibition (ZOI) and Minimum Inhibitory Concentration (MIC) experiments. The least efficient substance, petroleum ether, had the highest rates of bacterial survival. At the highest concentration tested, PHMB consistently showed a 100% death rate, demonstrating its strong antibacterial properties. The importance of PHMB as a standard for antibacterial efficacy is further emphasized by the study.

The literature survey highlighted the historical and traditional use of *T. occidentalis* in herbal medicine, particularly for its antimicrobial properties. Previous studies have documented the presence of various bioactive compounds in *T. occidentalis* that contribute to its medicinal properties. This background provided a foundation for the current study's focus on evaluating the antibacterial efficacy of its extracts.

The characterization of phytochemicals in *T. occidentalis* extracts revealed the presence of several bioactive compounds, including flavonoids, terpenoids, and phenolic acids. These compounds are known for their antimicrobial properties and likely contribute to the observed antibacterial activity against *Bacillus cereus*. The methanol extract, in particular, was rich in these phytochemicals, correlating with its higher efficacy at lower concentrations.

The characterization report detailed the methods used to identify and quantify the phytochemicals present in the extracts. Techniques such as GC and GC-MS were employed to analyse the chemical composition. The petroleum ether extract, while less effective, still contained some active phytochemicals, albeit in lower concentrations.

This investigation reveals the capability of methanol, chloroform, and petroleum ether extracts, as well as PHMB, to effectively inhibit the growth of *Bacillus cereus* bacteria. The study underscores the potential of *T. occidentalis* extracts as natural antibacterial agents, with methanol extract being particularly promising. Further research, including a deeper understanding of the underlying mechanisms, is necessary to fully explore the potential and practical applications of these substances in the fight against bacterial infections.

5.2 Future Recommendations

Based on the optimistic outcomes of this study, it is recommended to explore various potential areas for future research and development.

In Vivo Studies- Conduct in vivo studies to validate the antimicrobial efficacy and safety of *T. occidentalis* extracts in animal models and Assess the pharmacokinetics and pharmacodynamics of the active compounds to understand their behaviour in biological systems.

Broader Spectrum Analysis- Expand the study to include a broader range of bacterial strains, both Gram-positive and Gram-negative, to evaluate the spectrum of antimicrobial activity.

Assess the effectiveness of the extracts against antibiotic-resistant strains to explore their potential in addressing antimicrobial resistance.

Compound Isolation and Characterization- isolate and characterize individual active compounds from *T. occidentalis* extracts to identify the specific components responsible for the observed antibacterial activity. Utilize advanced techniques such as nuclear magnetic resonance (NMR) and mass spectrometry (MS) for compound identification and structural elucidation.

Formulation Development-Develop pharmaceutical formulations incorporating *T. occidentalis* extracts for topical or systemic application. Evaluate the stability, efficacy, and safety of these formulations in preclinical and clinical trials.

Toxicological Studies-Conduct comprehensive toxicological studies to assess the safety profile of the extracts and their individual compounds

Exploration of Additional Pharmacological Activities-Investigate other potential pharmacological activities of *T. occidentalis* extracts, such as anti-inflammatory, antioxidant, and anticancer properties.

Industrial Applications- Explore the potential use of *T. occidentalis* extracts in various industries, such as food preservation, cosmetics, and agriculture, due to their antimicrobial properties. Develop sustainable extraction and production methods to facilitate large-scale application and commercialization.

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